STUDY ON *PASTEURELLA MULTOCIDA* INFECTION IN CATTLE AND RABBITS

By

Mortada Mohammed Abbakar

B.V. Sc

University OF Khartoum (2002)

Supervisor: Dr. Khalid Mohammed Sulieman

Department Of Microbiology

University Of Khartoum

A thesis submitted in fulfillment for requirement of the degree of M.V.Sc.

Department of Microbiology

Faculty of Veterinary Medicine

University of Khartoum

2007

PREFACE

This work was carried out at the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, under the supervision of Dr. khalid Mohammed Suleiman.

Dedication

To my dear …..

Mother

Brothers and Sisters

With Deep love

ACKNOWLEDGMENT

First of all my thanks and prayers to almighty, the beneficent, the merciful, for giving me health and power to accomplish this work. When I wish to express my indebtedness and sincere to my supervisor Dr. khalid Mohammed Suleiman for their keen guidance, valuable assistance, advice encouragement. Indeed, their generous help and support is greatly appreciated.

I wish to express my lovely family, friends and colleagues. Thanks are also extended to technicians, the laboratory assistants and labors in the department of microbiology mainly Fawzia, A/Aziz, Mona, Elyas, Hashim, Saeed and Hassan

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The aim of this study was to compare the efficacies of systemic and mucosal immune responses to comparative protective preparations from *Pasteurella multocida* administered to rabbits and cattle.

Rabbits groups were immunization intranasally and subcutaneously with a bacterin, cell lysate and toxoid prepared from cultures of *P. multocida* serotype B: 6, while cattle groups were immunized intranasally and subcutaneously with the heamorrhagic septiceamia bacterin only. A mixture of freund incomplete adjuvant and pea-nut oil was used as an adjuvant to deliver the vaccine preparationfrom *P. multocida*.

Subcutaneous immunization of rabbit groups with the bacterin, cell lysate and toxin of *P. multocida* serotype B: 6 induced protection in rabbits. The protection was demonstrated in serum samples collected from immunized animals and examined for antibody titer by the indirect haemagglutination test IHA. The highest titers were obtained in the group that received the bacterin vaccine and the lowest titers were shown the group vaccinated with the cell lysate. Protection of immunized rabbits was further proved by challenge inoculation of the animals with viable *P. multocida*. The survival rate in all groups vaccinated subcutaneously was 100%.

Mucosal protection in rabbit groups immunized intranasally with the three vaccine preparation was measured by the indirect heamagglution testing of nasal lavages and ultimately by challenge exposure to viable *P. multocida*. Results of the IHA showed that the

highest titers were recorded in the bacterin-vaccinated group. Challenge inoculation in these groups revealed a 100% protection only in the bacterin-vaccinated group.

Cattle groups were immunized with a bacterin vaccine to group cattle via the subcutaneous and intranasal routes. The level of the antibody immune response in these two groups and a control group that received the bacterin vaccine without the adjuvant was measured by the IHA. Similar levels of protection were obtained in cattle groups vaccinated subcutaneously with two different bacterin doses. The mucosal level of antibodies in the intranasally vaccinated group was less compared to subcutaneously vaccinated cattle.

This study concluded that intranasal immunization of rabbits and cattle with *P. multocida* preparations induced a protective immune response against *P. multocida*.

ملخص الأطروحة

الهدف من هذه الدراسة هو مقارنة فعاليات الاستجابة المناعية الجهازية والمخاطية عن طريق مولدات الضد معدة من بالباستيوريلا المتعددة القتل في الأرانب والأبقار .

مجموعات الأرانب حصنت عن طريق الأنف وتحت الجلد بلقاح التسمم الدموي ,الخلايا المحللة والسم المضعف المعد من تزريع بكتيريا الباستيوريلا متعددة القتل ذات النمط المصلي ب 6. بينما مجموعات الأبقار حصنت عن طريق الأنف وتحت الجلد بواسطة لقاح التسمم الدموي فقط. استخدم المساعد المناعي المكون من خليط مساعد الفروند الغير كامل مع زيت الفول السوداني لتسليم مولدات الضد المعدة من بالباستيوريلا متعددة القتل.

التحصين تحت الجلد بواسطة لقاح التسمم الدموي ,الخلايا المحللة والسم المضعف المعد من بكتيريا الباستيوريلا متعددة القتل ذات النمط المصلي ب 6 يحدث مناعة في الأرانب. الحماية وضحت في عينات السيرم التي جمعت من مجموعات الحيوانات المحصنة ثم اختبر معيار الأجسام المضادة باختبار تلازن الدم الغير مباشر . اعلي المعايير سجلت في المجموعة التي حصنت بلقاح التسمم الدموي واقل المعايير سجلت في المجموعة التي حصنت بالخلايا المحللة الحماية في الأرانب المحصنة برهنت الحماية إلي حد ما بجرعة التحدي ببكتيريا بالباستيوريلا المتعددة القتل . معدل البقاء في الأرانب المحصنة عن طريق تحت الجلد كان 100%.

المناعة المخاطية في مجموعات الأرانب المحصنة داخل الأنف باللقاحات الثلاثة قيست بالتلازن الدموي الغير مباشر لغسيل الأنف ثم اختبرت بجرعة التحدي ببكتيريا الباستيوريلا المتعددة القتل . نتائج اختبار التلازن الدموي الغير مباشر أعطت معايير عالية في المجموعة المحصنة بلقاح التسمم الدموي التي سجلت معدل حماية 100% عند أعطا جرعة التحدي في المجموعة المحصنة بلقاح التسمم الدموي

استخدم المساعد المناعي المخاطي لتسليم لقاح التسمم الدموي لمجموعات الأبقار عن طريق تحت الجلد وداخل الأنف مستوي الأجسام المضادة في الاستجابة المناعية في المجموعتين ومجموعة التحكم التي استقبلت لقاح التسمم الدموي بدون المساعد المناعي

ثم اختبرت بواسطة اختبار تلازن الدم الغير مباشر . إعطاء جرعتين مختلفتين من لقاح التسمم الدموي عن طريق تحت الجلد أعطي مستوي حماية متشابه. مستوي الأجسام المضادة في مجموعة الأبقار المحصنة داخل الأنف اقل عند مقارنته مع مجموعة الأبقار المحصنة عن طريق تحت الجلد.

خلصت الدراسة إلي إن التحصين داخل الأنف في مجموعات الأبقار و الأرانب بالمستحضرات المعدة من بكتريا الباستيوريلا متعددة القتل تعطي استجابة مناعية ضد بكتيريا بالباستيوريلا متعددة القتل.

CHAPTER ONE
INTRODUCTION

Haemorrhagic septicaemia (HS) is a peracute, contagious and highly fatal septicaemic disease of cattle endemic nature in Asia and Africa (Bain, 1957). *P. multocida* is the primary causative agent of (HS) in cattle and buffaloes, (Thomson *et al*., 1975). Under stresses, such as shipping and experimental handling, various serotypes of *P. multocida* may replicate rapidly, causing diseases such as pneumonia, conjunctivitis, and septicaemia (Flatt, 1974), and atrophic rhinitis (DiGiacomo, 1989). The pathogen is highly contagious and is readily transmitted through direct physical and aerosol contact (DiGiacomo, 1987), making eradication difficult.

The serotypes causing HS in Sudan serotypes B: 6 and E: 6 were isolated and identified from cases of HS in cattle by (Shigidi and Mustafa, 1979). The disease was reported in camels by (Hassan and Mustafa, 1985) and serotype B: 6 was identified as the causative agent of the disease. Economic impact of HS is firmly rooted in many African and Asian countries with regular sporadic or massive seasonal outbreaks resulting in losses. HS as a killing disease has become the most serious disease of livestock.

Antibiotics have been only partially successful in controlling infection, since they do not completely eliminate the bacterium (Jaslow, 1981) and, like many other bacteria, *P. multocida* has developed resistance to some of the commonly used antibiotics (Watts,

1994). Moreover, antibiotics are only a temporary solution to the problem because infection usually recurs within a short period of time following treatment (Gaertner, 1991). Another potential means to control pasteurellosis is through vaccination. In the Sudan HS in controlled by vaccination with a bacterin grown in afermenter under the optimizal physical growth conditions,(temperature 37.5ºC, pH 7.4 and stirring 300 rpm) determinated by Elbashir, (1993).

Since *P. multocida* infections a primary occurs in the upper respiratory tract, the mucosal immune response is likely to be an important defense mechanism. Secretory IgA (S-IgA) antibodies are abundant in mucosal secretions and function to inhibit microbial adherence to epithelial cells (Mestecky, 1987). S-IgA is preferentially induced following mucosal immunization ; thus, the production of S-IgA following intranasal (I.N) vaccination could help to prevent bacterial colonization and subsequent infection.

The objectives of this study were:

(i) to evaluate the efficacy of intranasal (I/N) versus subcutaneous (S/C) administration of the vaccines in stimulating protective immunity against *P. multocida* infection, following vaccination of rabbits and cattle with a bacterin, cell lysate and toxoid preparation from *P. multocida*.

(ii) to test the safety of *Nigella sativa* oil, a mixture pea-nut oil and Freund's incomplete adjuvant as mucosal adjuvants for delivery of *P. multocida* vaccine preparations.

CHAPTER TWO
LITERATURE REVIEW

2.1. Taxonomy of *P. multocida*

Pasteurella(P.) multocida belongs to the family *Pasteurellaceae* which contains the genera *Pasteurella*, *Actinobacillus*, *Gallibacteria*, *Haemophilus*, *Lonepinella*, *Mannheimia*, and *Phocoenobacter*. These genera share a number of common features and some organisms have been reclassified within these genera following deoxyribonucleic acid hybridization studies and 16S rRNA sequencing Quinn, *et al*., (2002), Hirsh, Maclachan and Walker, (2004).

2.2. Morphology and cultural characteristics

P. multocida was described as gram-negative, non motile, encapsulated and facultative anaerobic bacteria. The cells are usually coccobacillary or short rods in cultures from sick animal tissues and show bipolar appearance in smears stained by Leishman and Geimsa stains, whereas strains of *P. multocida* from apparently healthy animals were often pleomorphic with occasional short filaments Carter, (1957). The capsule of *P. multocida* type A is made of hyaluronic acid, type D capsule is of heparin and the capsule of type F is made of chondroitin, Hirsh, *et al*., (2004).

2.3. Biotypes of *P. multocida*

Five biovars of *P. multocida* were proposed by Carter, (1967) designated as biovar a (the mucoid biovar), biovar b (the haemorrhagic septicaemia biovar), biovar c (the porcine biovar), biovar d (the feline biovar) based upon hyaluronidase decapsulation, acriflavine folliculation, colonial iridescence, fermentation pattern, mouse pathogenicity, host predilection and serologic and immunologic characteristic. The fifth biovar is designated E (deer biovar) on the bases of its acid reactions in trehalose, sorbitol and mannitol and their failure to produce hydrogen sulphate Frederiksen, (1973).

2.4. Antigenic structure of *P. multocida*

Twenty eight heat-labile and 9 heat-stable antigenic components from disintegrated serotype A *P. multocida* from rabbits were demonstrated by electrophoretic analysis Stepkowskii and Zarzycki, (1986). Lipopolysaccharide (LPS) comprised the major component that resulted from chemical or physical treatment of *P. multocida* to identify their antigenic structure.

2.5. Serotypes of *P. multocida*

Different methods employing a wide range of antigenic components and cellular fractions were adopted to serotype *P. multocida*. Roberts, (1947) classified strains of *P. multocida* into 4 serological types I, II, III and IV by the passive mouse protection tests in which mice were passively immunized by antisera prepared against one strain and counter challenged by the homologous or heterologous

virulent cultures of the strain.

The colonial morphology and antigenic behavior of variants of *P. multocida* employing the S, R and M designation of Carter, (1957), using the acriflavine test revealed a characteristic reaction when freshly isolated type D *P. multocida* were subjected to the test. The test rapidly identified the non-immunogenic cultures of *P. multocida* together with the untypable ones. In 1955, Carter described a haemagglutination test for identifying *P. multocida* utilizing specific capsular antigens and capsular hyaluronic acid extracted by heating the cells at 56°C for 30 minutes in normal saline. These were adsorbed to human group (O) erythrocytes and positive results were detected by visible agglutination. The test recognized four different serological groups designated A, B, C and D and failed to allocate non encapsulated cultures to any specific group. When compared to Roberts type I, II, III & IV they were found to be identical to type B, A, C and D respectively. Carter, (1967) identified a strain of *P. multocida* isolated from cattle with an acute septicaemic disease which was different from that caused by serotype B *P. multocida* in cattle and buffaloes and designated it as serotype E. His serotype C gave inconclusive results in further studies and was dropped as a distinct serological group. Rimler and Rhoades, (1986) identified an additional capsular serogroup designated serogroup F among *P. multocida* isolates from turkeys in USA with somatic serogroups 1, 3, 7 and 12.

Namioka and Murata (1961) studied the antigenic relationships between the somatic antigens (O-Ag) of *P. multocida* prepared by treating cultures of the organism with normal hydrochloric acid (N-

HCL) and divided them into common and specific antigens. Cultures of type A and D were divided into two groups according to their O-Ag. Subculturing of the organisms did not change the O-Ag into the rough form even if it was repeated for a year or more. They distinguished 6 somatic groups (1-6) among 24 strains of *P. multocida* from different parts of the world by means of absorption tests and consequently 6 serotypes were specified by combining their capsular types and somatic groups. On studying 156 cultures of *P. multocida* obtained from different countries, two years later Namioka and Bruner, (1963), demonstrated 10 somatic groups designated 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 identified 12 serotypes when they were correlated to Carter´s capsular types ; 5 O-groups (1, 3, 5, 7 and 9) being distinguished among cultures of type A, an other 5 O-groups (1, 2, 3, 4 and 10) among type D and a single O-group (6) in type B. A highly pathogenic strain of *P. multocida* (strain P-1059) identified as group 8 was not found to be identical with other group 8 strain (strain 147) and it was then represented as 8b and the later as 8a. Strain Bunia 11 of Carter's serotype E that was recovered from cattle with HS in Congo belonged to O-group 6 as did the serotype E strain of Carter that came from Chad.

Namioka's agglutination test and Heddlestons gel diffusion precipitin test employed by Shigidi and Mustafa, (1980) to study the serological inter-relationships of 24 strains of *P. multocida* represented by 11 strains, each representing a different serogroup, and 13 strains from cattle with HS agreed in their results indicating that both tests were probably employing the same antigen.

2.6. Pathogenicity of *P. multocida*

Haemorrhagic septicaemia (HS) of cattle and buffaloes was reported in Africa, Asia, the Middle East and Southern Europe and it appeared in the form of shipping fever in the Western hemisphere Bain, (1963); Geering, (1984). HS of cattle was particularly associated with water courses, deltas and wet farming areas, besides, it could also occur in dry areas in some countries Geering, (1984). Beef cattle grazing effluent irrigated pastures during wet summer months with excessive rain fall and unusual cold weather in the preceding month experienced a severe outbreak of HS and the young animals were the most affected. The animals manifested the acute form of the disease with pyrexia, tachycardia and bradycardia, excessive salivation, extreme pallor of the mucous membranes and some animals showed submandibular and facial oedema and bilateral corneal opacity together with the enlargement of the parotid and submandibular lymph nodes as well as meningitis which was almost a constant feature among all affected animals Lane, *et al*., (1992). In addition there might be development of warm painful swelling about the throat, dewlap, briskets or perineum with submucosal petechiae Krishna and Kaushik, (1965). Francis, *et al*., (1980) recorded high fever, depression, anorexia, increased pulse and respiratory rates, serous nasal discharge and excessive salivation in cattle infected with type E *P. multocida* in Zimbabwe. Septicaemic pasteurellosis was found responsible for heavy losses chiefly in low-lying areas of Southern Asia when animals were exposed to wet and chilly weather or exhausted by heavy work Bain, (1963).

The organisms were isolated as primary etiological factors of the disease Carter, (1957); Martinez. *et al*., (1987),

Camel pasteurellosis was reported by Hassan and Mustafa, (1985), who isolated *P. multocida* organisms from the blood, exudates and lyphatics of affected camels. Bain (1963) enlisted septicaemic camel pasteurellosis in his report that described the prevalence of the disease in Southern Asia. Serotype B *P. multocida* which was virulent to rabbits and calves was primarily isolated from camels that had died of an acute disease in Sudan Hassan and Mustafa, (1985).

Sheep pneumonia due to *P. multocida* was distinguished and the organisms were principally demonstrated from sheep Kadymov *et al.* (1987).

Fowl cholera, a septicaemic disease of avian species, was reported to be predominantly caused by *P. multocida* serotype A Carter, (1959). Four capsular serotype (A, B, D and F) were distinguished among avian strains of *P. multocida* isolated in the USA Rimler and Rhoades, (1986).

2.6.1. Pathophysiology:

Local: *P multocida* infection most commonly presents as an infection complicating an animal bite or injury. Complications include rapidly progressive cellulitis, abscesses, tenosynovitis, osteomyelitis, and septic arthritis. The latter two are particularly likely in cat bites because of the small, sharp, penetrative characteristics of feline teeth.

Respiratory: *P multocida* causes upper respiratory tract infections, including sinusitis. It also rarely causes lower respiratory tract infections (primarily in

patients with underlying pulmonary disease), including pneumonia, lung abscess, and empyema.

Cardiovascular: *P multocida* may cause endocarditis, *myocardium arrthymia,* vascular graft infections, central venous catheter infections, and pericarditis.

Central nervous system: *P multocida* is an uncommon cause of meningitis, subdural empyema, and brain abscess. *P multocida* meningitis has been related to cat licks or bites. These diseases typically occur in persons at the extremes of age.

Gastrointestinal: *P multocida* rarely causes gastrointestinal problems. Such problems may include appendicitis, hepatosplenic abscesses, renal abscesses, and peritonitis. Patients with peritoneal dialysis-catheter–related peritonitis have been reported.

Ocular: Periocular abscess, conjunctivitis, corneal ulcers, and endophthalmitis have been reported.

Genitourinary tract: Pyelonephritis, renal abscess, epididymitis, and cervicitis are reported rarely, Thomas, (2006).

2.7. Disease

2.7.1. Pasteurellosis

Pasteurellosis is a common name of the different diseases of domestic and wild animals which is caused by or associated with different members of the genus *Pasteurella* Carter and Bain, (1960).

The disease was first described by Bolliger, (1878) in cattle, followed by Pasteur, (1880) who described the fowl cholera causative agent, and Kitt, (1885) who isolated the organism from septicaemia outbreaks in different animals. Hueppe, (1886) proposed the collective name for these similar diseases as haemorrhagic septicaemia. Some diseases caused by *P. multocida* shall be mentioned here. *P. multocida* has a commensal existence in the upper respiratory and digestive tract of healthy animals and is a frequent secondary invader or opportunist in a number of pathogenic processes.

P. multocida is a primary causative agent of fowl cholera and haemorrhagic septicaemia (HS) in cattle and buffaloes or secondary invader in pneumonia of cattle, Thomson, *et al*., (1975), swine, sheep, goat and other species as well as its frequent involvement in bovine shipping fever and enzootic pneumonia of pigs. In addition to these diseases, the organism was recovered from a wide range of sporadic infections in many species and was said to be one of the causes of the snuffles in rabbits and severe mastitis of sheep and cattle. Dogs, cats and other species frequently harbour the organism in their mouths as commensal, Smith, (1955) and consequently man and animal may be infected from their bites Bailie, *et al*., (1978). *P. multocida* is considered also as opportunist or secondary invader in internal infection in human Hubbert and Rosen, (1970)

2.7.2. Haemorrhagic septicaemia (HS)

Haemorrhagic septicaemia (HS) is a peracute, contagious and highly fatal septicaemic disease of both wild and domestic animals,

with endemic nature in most parts of tropical and subtropical Asia and Africa Bain, (1957). Haemorrhagic septicaemia has been defined as a special kind of primary pasteurellosis, Bain, (1954). HS is caused by *P. multocida* serotypes B: 6 and E:6 Banerji, (1953). Serotype B:6 is the causative agent of the disease in Asia, while serotype E: 2 was recovered from the disease only in Africa Carter, (1984). Few African countries, notably Egypt and Sudan, have reported both serotypes, Shigidi and Mustafa, (1979).

2.7.2.1. Geographical distribution

Haemorrhagic septicaemia (HS) has a worldwide distribution and is almost endemic in the African and Asian continents. In Africa it was reported in most countries Annosa, *et al*., (1975). The disease was found across Asia from the middle east region to south eastern Asian countries including Indonesia and Philippines Anonymous, (1979). Japan was free from the disease since 1954, Carter, (1984). No confirmatory reports exist on the presence of the disease in Europe with the exception of Italy, east European countries and one sporadic case of haemorrhagic septicaemia in fallow deer in the United Kingdom Johnes and Hussaini, (1982). The disease was reported in the United States Carter, (1982). The disease has not been reported in Australia and Canada Bain, (1963). HS which is sometimes mixed with pneumonic pasteurellosis of cattle, is of great significance in Mexico, central American countries including Costa Rica, Guatemala and south America Carter and De Alwis, (1989).

2.7.2.2. Economical importance

Haemorrhagic septicaemia is firmly rooted in many African and Asian countries with regular sporadic or massive seasonal outbreaks resulting in tremendous losses. Haemorrhagic septicaemia as a killing disease has become the most serious disease of livestock. Great losses in buffaloes which is a species of vital importance in the rural economy of Asian region, and cattle in many countries were reported Bain, (1963). The estimated losses were ranging between 20-98%. In thailand 10,000 annual deaths were reported, mostly useful draft and milk producer buffaloes. In India the annual losses ranged between 30,000-50,000 deaths in cattle. In Sri Lanka the annual losses in buffaloes were estimated to be about 15.23% and in cattle about 8.6% De Alwis, (1981) the estimated annual losses in this region were reported to be 90 million Rupees De Alwis, and Vipulasiri, (1980). Heavy losses in cattle with high mortality rate was also reported in Zambia, Francis *et al*., (1980).

2.7.2.3. Epidemiology

Multi-integrated factors were believed to govern the incidence of haemorrhagic septicaemia. A definite interaction of clinical, immunological and epizootiological factors with the disease pattern, De Alwis, (1984).

2.7.2.3.1. Host

A wide spectrum of wild and domestic animal species were susceptible to HS. Cattle and buffaloes Bain, (1963). Buffaloes are more susceptible than cattle Anonymous, (1933, 1959). The high

susceptibility of rabbits and mice, and a lesser degree susceptibility of guinea pigs, pigeons and horses were confirmed experimentally by Bain, (1963). A variable susceptibility of sheep, goats and fowl, Anonymous, (1933, 1959).

2.7.2.3.2. Carrier status

The healthy animals carrying the pathogen in their nasopharynx and tonsils De Alwis, (1990), play a great role in the disease by direct contact or through contamination of soil, water and pasture, and hence transmission of infection through inhalation or ingestion route. The carriers are of great importance in (HS) outbreaks in many countries Gupta, (1962). Variation in degrees of carrier and its significance in the epidemiology picture of the disease were reported De Alwis, *et al*., (1986a).

2.7.2.3.3. Naturally acquired immunity

This phenomenon was reported in enzootic areas Dhanda, (1959). It was assumed that it may be due to the natural exposure of the animal to arrested infection or to virulent organisms, and in both cases there are no clinical signs of the disease due to the level of immunity. The significant role in the epizootiology of (HS) among cattle and buffaloes should be considered, Bain, (1954).

2.7.2.3.4. Environmental factors

Clear demarkation into wet, humid and dry climate greatly contributes to the disease Baba, (1984). The epidemiological studies reported by many workers showed the intimate relation of the wet

season to HS outbreaks, De Alwis, (1981)

2.7.2.3.5. Predisposing factors

Parasitic, viral and bacterial infections, and overworking specially in buffaloes, transport, successive change in nutrition, management, and temperature with the changing of seasons, and the mode of husbandry were reported as predisposing factor, Annosa, and Isoun, (1975).

2.7.2.3.6. Other factors

In enzootic areas a lower mortality rate among old animals more than two years was observed, while in non-enzootic areas animals are found to be susceptible, Baba, (1984). Other factors like the animal species play an important role, e.g. buffaloes more susceptible than cattle, De Alwis, (1981).

2.7.2.4. HS in the Sudan

The disease is endemic in Sudan and was reported nearly from all parts of the country. The disease was reported in Blue Nile Province (1933), Kassala (1939), Northern kordofan (1943), and Upper Nile (1947) (Sud. Vet. Serv. 1933, 1939, 1943, and 1947). Serotypes B: 6 and E: 6 were isolated and identified from natural cases of HS in cattle by Shigidi and Mustafa, (1979).

The disease was reported as an acute Pasteurellosis in camels in the Blue Nile province by Hassan and Mustafa, (1985) and serotype B: 6 was identified as the causative agent of the disease. Disease outbreaks usually occur after of the rainy season. Changes of weather

from dry summer which is characterized by poor range conditions to a high humid moderate temperature with abundant good pasture during the rainy season was believed to be the major stress factors that enhances the appearance of the disease.

2.7.2.5. Clinical signs

The disease is characterized by rapid course, high fever, salivation, conjunctivitis, lacrimation, cessation of rumination and dullness followed by sudden death in preacute condition. In acute cases dysponea, painfull groans, respiratory distress, oedematous swelling in the head-throat-brisket region and fore limbs followed by recumbency at later stages were reported by Bastianello, and Jonker, (1981).

Post-mortem lesions most animals succumbing to HS typically show swelling of the neck due to severe blood-tinged oedema. There are abundant petechial haemorrhages involving many tissues, and particularly serosal membranes. The thoracic, pericardial and abdominal cavities may contain serosanguinolent fluid. The lungs are congested and notably oedematous. These lesions are similar to those observed in severe sepsis.

2.7.2.6. Diagnosis of HS

A tentative diagnosis is very essential for combating the spread of the disease through both treatment of clinically diseased individuals and implementation of usual control measures Carter, *et al*., (1972). Clinical diagnosis is possible to conduct on the basis of the individual

and herd history, characteristic clinical signs, morbidity and mortality patterns, together with the species and age susceptibility.

2.7.2.6.1. Microscopy:

Direct examination of blood smears stained by Gram stain reveal small gram-negative, coccobacilli with characteristic bipolar staining.

2.7.2.6.2. Cultural characteristic

Colonies *P. multocida* on blood agar after inocubation for 24 to 48 hours at 37ºC are round, grayish and some strains produce large mucoid colonies.

2.7.2.6.3. Serological tests

Numerous serological tests had been described for the detection of *P. multocida* antibodies in naturally immune as well as in vaccinated animals. These tests include the tube agglutination test and the serum bactericidal test Bain, (1963). The original indirect haemagglutination test (IHA) of Carter, (1955) was modified by Carter using formalinized human O cells and later by Sawada *et al*. (1982) using glutaraldehyde treated sheep erythrocytes. Recently an enzyme linked immuno-sorbent assay (ELISA) for identification of *P. multocida* was developed by Dawkins *et al*., (1990), a useful diagnostic technique was recommended by O.I.E. (1990).

2.7.2.6.4. Agglutination test

2.7.2.6.4.1. Rapid slide agglutination test (capsular typing)

A single colony is mixed with a drop of saline on a slide, a drop of antiserum is added, and the slide is warmed gently. A coarse,

floccular agglutination appears within 30 seconds. Old cultures may give a fine, granular agglutination that takes longer to appear.

2.7.2.6.4.2. Indirect haemagglutination test (capsular typing)

This was originally performed using antigen-sensitized human type (O) red blood cells (RBCs) Carter, (1955) but more recently sheep (RBCs) have been used. The antigen was prepared as follows; Bacteria cultured on blood agar and incubated overnight at 37°C. The growth was harvested in 3 ml normal saline containing 0.3% formalin. This suspension was then heated at 56°C for 30 minutes, centrifuged 3000 rpm for 15 minutes at 4°C, and the clear supernatant was frozen at -20°C. This was used as the antigen extract. Sheep blood was collected aseptically into an anticoagulant and centrifuged at 500 rpm for 10 minutes. The packed red blood cells (RBCs) were washed three times in sterile normal saline. The antigen extract described above was used to sensitize the red blood cells (RBCs) or absorbed on to the red blood cells (RBCs). This is done by adding 15 volume of the antigen extract to the red blood cells (RBCs) and incubating the mixture for one hour at 37°C with frequent shaking. The sensitized red blood cells (RBCs) are recovered by centrifugation, washed three times in sterile normal saline, and made up to a final 1% suspension in normal saline Carter, (1955).

2.7.2.6.4.3. Agglutination test (somatic antigen)

The somatic 'O' antigen was prepared by a method similar to that described previously for the IHA test Namioka and Murata, (1961). A 6-8 hour test culture was seeded on to casein sucrose yeast (CSY)

blood agar and incubated overnight. The growth is harvested in 2-3 ml of physiological saline containing 0.3% formalin , and centrifuged at 3000 rpm for 15 minutes at 4°C (or 1200-1500 rpm for 30-45 minutes at room temperature). The deposited bacteria are resuspended in 25 ml normal HCl saline (0.85% saline in a normal HCl solution) to give an opacity approximately equivalent to Brown's opacity tube No. 6, and incubated overnight. The suspension is again centrifuged, the supernatant fluid is discarded, and the cell residue is washed three times successively in phosphate buffered saline (PBS) at pH 5.0, 6.0 and 7.0, respectively. Finally, a suspension of the residual cells, equivalent to Brown's opacity tube No. 6, is prepared in PBS at pH 7.0. Any suspensions that autoagglutinate should be discarded.

Antisera are prepared against whole bacterial cell suspensions of the reference strains B:2 (Asian HS), E:2 (African HS) and B: 11 (Australian 989, non-HS). The agglutination test is carried out on a slide and the test antigen is used against the three types of sera. A fine granular agglutination indicates a specific somatic agglutination. The test carried out against the standard antigens will facilitate reading and interpretation. When nonspecific partial agglutination occurs, the test is carried out with tenfold dilutions of the serum against the test and standard antigens will help to identify somatic antigen.

2.8. Vaccine

Vaccine is a product which may be a suspension of living or dead cells, or an antigenic fraction of cells, which when injected with or without adjuvant enhance the immunity production in the animal′s

body, Bain, (1954).

2.8.1. Type of vaccines

2.8.1.1. Killed vaccine

The antibody response against killed vaccines depend on the method of cultivation, way of preparation and killing, presence of adjuvants, degree of antigenic variation, route of administration and dose.

2.8.1.2. Attenuated vaccine

The attenuated live vaccines are prepared by passaging the organism in non-ordinary host or by growing in unfavourable conditions. Attenuation trials of HS vaccine were achieved by passage in pigeons. The use of live attenuated *Pasteurella* organisms as vaccine to prevent the disease caused by *P. multocida* was first tried against fowl cholera by Pasteur, (1880) . Recently the attenuated live vaccines against fowl cholera were reported by Bierer, (1969).

2.8.1.3. Toxoid vaccine

Toxoid vaccine was proved useful against atrophic rhinitis caused by or associated with toxigenic strains of *P. multocida* Bording and Riising, (1986). Kobish and Pennigs (1986) reported a protection against atrophic rhinitis by vaccination with *P. multocida* toxin.

2.8.1.4. Other vaccine

Other types of vaccines were tried in small experimental scale with a quite variable degree of protection. Strong immunity was found on using germ free filtrate of inflammatory exudates by Gochenour,

(1924).

2.9. Vaccination

Vaccination is to induce the maturation of cells involved in the specific removal of a pathogenic agent and to maintain clones of memory cells specific for the target antigens in the circulation and in the secondary lymphoid organs. The important contribution of B cells as antigen presenting cells to the activation of T cells has been reported Ron, *et al.*, (1981).

In all countries where HS occurs, vaccination is adopted as the method of control. A variety of antigen preparations were developed and used to confer protection in animals against *P. multocida* infections. These included inactivated whole cell preparations (bacterins), life whole cell cultures, aggressins, and extracted cell fractions and components. Varying degrees of effectiveness were recorded throughout the history of the use of bacterins as immunogens against different *P. multocida* infections in different host species Joseph and Hedger, (1984); yet, the use of adjuvants improved the immune response to some bacterins Yadav and Ahooja, (1986). The fact that *P. multocida* infections were often associated with other precipitating factors had, however, necessitated the use of combined vaccines Confer, *et al*., (1985).

Aggressins and antisera were used to protect cattle against respiratory disease Mosier, et al., (1989). Extracts of bacterial components including capsular extracts Nagy and Penn, (1976), LPS and proteins Lutenbergh, (1986), saline purified CHO-protein fractions Syuto and

Matsomoto, (1982) and chaotropically-extracted antigens e.g. KSCN extract Lu, *et al*., (1987), all had yielded superior beneficial results in animals challenged with homologous and sometimes heterologous *P. multocida* organism.

In Sudan annual vaccination using a bacterin produced by the local Veterinary Research Laboratory, from local bacterial isolates of *P. multocida* serotypea B : 6 and E : 6. The bacterin vaccine was produced in fermenter under optimized physical growth conditions, temperature 37.5ºC, pH 7.4 and stirring 300 rpm, reported by Elbashir, (1993).

2.10. Immunity against *P. multocida*

It is generally believed that, the type of immunity developed against *P. multocida* is mainly antibody mediated. Nagy and Penn (1976) reported that antibody level in vaccinated cattle determined by passive mouse protection (PMP) test indicated that immunity to HS is exclusively antibody mediated. The duration and the magnitude of these responses are affected by many factors such as virulence, potency of the antigen, the dosage, and the method of induction and the age of the animal.

The mechanism of the protection effect of antibody is shown by slowing of the bacterial multiplication with prolongation of the lag phase, aided possibly by conglutinin. The previous assumption that the major protection mechanisms against HS were due to specific opsonin and complement factors was explained by Dawkins *et al*., (1991).

2.10.1. Types of immunity to HS

Types of immunity to HS are recognized as naturally acquired, artificial active, or passively inducible.

2.10.2. Active natural immunity

In endemic areas, however, regular outbreaks occur mostly during the wet season, depending on an interaction between a number of factors (burden of infection, host defense mechanism, immunity level, etc). Some animals succumb to clinical disease while others will develop what is described as an arrested infection leading to a naturally acquired immunity De Alwis, (1987). Thus in endemic areas, the adult population through successive exposures acquire natural immunity and only small number of hitherto unexposed animals will remain susceptible at each outbreak. It is assumed that morbidity and mortality due HS in a given population largely depend on the proportion of the immune to non-immune animals and therefore the phenomenon of naturally acquired immunity is responsible for the different patterns of morbidity and mortality in endemic and non endemic areas .

2.10.3. Active artificial immunity (Vaccination)

Vaccination is generally accepted as the best mean of control against

Pasteurellosis and vaccines in different forms are readily available throughout the world.

2.10.4. Passive artificial immunity

Mosier *et al.* (1989) reviewed the evolution of vaccines for bovine pneumonic Pasteurellosis, where they stated that, the use of HS antisera to induce passive immunity to HS was practiced since 1918. The use of HS antisera was found to be of value when given before shipment to control shipping fever.

2.11. Mucosal immunity

The mucosal surfaces of the gastrointestinal and respiratory tract represent the principal portals of entry for most pathogens. Most external mucosal surfaces are replete with organized follicles and scattered antigen-reactive or sensitized lymphoid elements, including B cells, T lymphocytes, T cell subset, plasma cells and a variety of other cellular elements involved in the induction and maintenance of immune response. The major antibody isotype in external secretions is secretory immunoglobulin A (S-IgA). Approximately 40 mg of IgA per Kg of body weight is secreted daily, especially from the gastrointestinal tract. It is, however, interesting that the major effector cells in the mucosal surfaces are not IgA B cells, but T lymphocytes of CD4 as well as CD8 phenotypes, it is estimated that T lymphocytes may represent up to 80% of the entire mucosal lymphoid cell population, Conley, (1987).

2.11.1. Common mucosal immune system

The immunologic network operating on external mucosal surfaces consists of gut associated lymphoid tissue (GALT), the lymphoid structures associated with bronchoepithelium and lower

respiratory tract (BALT), occular tissue, upper air way, salivary glands, tonsils and nasopharynx (NALT), larynx (LALT), middle ear cavity, male and female genital tracts, and the mammary glands. The organized lymphoid follicles in the GALT and BALT are considered the principal inductive sites of mucosal immune response, Staats, (1994).

2.11.2. Gut and bronchoepithelium associated lymphoid tissues

A substantial body of information has been generated with peters patches and other organized lymphoid follicles in the gut-associated lymphoid tissue (GALT), including the appendix Dasso, (1997) and the bronchoepithelium associated lymphoid tissue (BALT) concerning the induction of mucosal immune responses and the development of systemic hypo-responsiveness following oral exposure to an antigen (oral tolerance) Javed, (1995). The common features of all inductive mucosal sites include an epithelial surface containing M cells, overlying organized lymphoid follicles, mucin producing glandular cells lymphocytes, plasma cells, dendritic cells, and macrophages. The mucosal epithelial cells express polymeric immunoglobulin receptor (pIgR) and secretory component, major histocompatibility complex (MHC), class 1 and 11 molecules, other adhesion molecules, and a variety of cytokine and chemokine receptors McGhee, (1999). The dendritic cells are present in different components of the common mucosal immune system, including the organized lymphoid tissue and the mucosal epithelium; these cells can be strongly associated with initiation of immune response and promote development of active

immunity Liu, (1993). Recent observations have suggested that dendritic cells are potent antigen-presenting cells (APC), and are critical in initiating primary immune responses, graft rejection, autoimmune disease, and generation of T-cell-dependent B-cell responses. The APC function is attributed in part to their ability to express costimulatory molecules (CD80 and CD86), and other accessory ligands necessary for up regulation or induction of tolerance Steinbrink, (1997).

2.11.3. Nasopharynx Associated lymphoid Tissue (NALT)

Nasopharynx associated lymphoid tissue NALT is well described and characterized in small laboratory animals. It is present as paired lymphoid aggregates in the floor of the nasal cavity at the entrance to the pharyngeal duct. In farm animals, no comparable aggregates are found at these sites, but isolated lymphoid nodules (ILF) have been described in horses and sheep.

2.11.4. Lymphoid tissues of the Waldeyer's Ring

In contrast to small laboratory rodents, the lymphoid tissues of Waldeyer's ring are well developed in farm animals and humans. Therefore, interest has centered on them instead of NALT. Lymphoid tissues of Waldeyer's ring guard the nasal, oral and auditory passages into the pharynx. They are formed by large aggregates of lymphoid nodules termed tonsils that occur constitutively at distinct anatomical sites in the pharynx of each species and ILF that vary in number. In the oropharynx, the lingual tonsil, the palatine tonsil and the tonsil of the soft palate are present; in the nasopharynx, the pharyngeal and tubal

tonsils are present. Tonsils are important for inducing immunity at mucosal sites. Some pathogens have, however, developed mechanisms to overcome tonsillar defenses and may use them as the port of entry, replication and colonization Huang, *et .al.,* (1990). Several pathogens are able to persist asymptomatically within the tonsils making the identification of carriers difficult in disease control and elimination. Therefore, tonsils are highly important tissues for diagnostic investigations of infectious diseases. The pharyngeal and tubal tonsils are the main targets for nasal vaccines which are attractive, because of the relative accessibility and high permeability of tonsils and the micro-environmental conditions with less acidic pH, lower levels of enzymatic activity and no ruminal digestion Stanley, *et al*., (2001). Although not widely used, nasal vaccination may provide a practical alternative to oral vaccination to induce mucosal immune responses. The intranasal inoculation of sheep with *Pasteurella hemolytica* caused a significant increase in the size of BALT, a significant increase in numbers of BALT structures and had a protective effect against colonization Zamri. *et al*., (1999).

2.11.5. Larynx-associated lymphoid tissue (LALT).

Some evidence suggests the existence of organized lymphoid tissue in the larynx in human, lymphoid aggregates have been observed at the laryngeal side of the epiglottis in >80% of infants and children younger than 22 months of age. In the follicular areas of the aggregates, most cells appears of B lymphocytes, with some CD4 lymphocytes in the germinal centers, and the inter-follicular areas

contain equal numbers of B and T cells. Other investigators have also observed scattered lymphocytes in the laryngeal epithelium. It remains to be determined whether larynx-associated lymphoid tissue (LALT) is a distinct physiologic entity or a pathologic reaction in response to local infection or other environmental insults, Tschernig, (1995).

2.11.6. Other mucosal immune systems

The tonsils, middle ear cavity, male and female genital tracts, mammary glands and salivary glands exhibit similar distribution of IgA and IgD immunocytes. In addition, scattered areas in the crypt epithelium of nasopharyngeal tonsils express secretary component. Another important feature of mucosal lymphoid tissue and the follicular germinal center is induction of the J-chain gene in some B cell subsets. Tonsillar germinal centers express a very high percentage of extra follicular immunocytes with J-chain expression. More than 90% of these immunocytes are of the IgA isotype Bernstein, (1999).

2.12. Mucosal adjuvants

Adjuvants have a complex mechanism of action as immunostimulators, delaying the absorption of vaccine and prolonging the antigenic stimulus to the antibody forming cells. A number of chemical agents have been tested to enhance the immunogenicity of mucosally administered antigens including cholera toxin, *E. coli*-labile toxin, actins, low-oil emulsion (MF59), lipid A, lysophosphatidyl glycerol and cytokines (IL-5). Such antigens in general induce only weak or insufficient immune responses when administered mucosally. Adjuvants influence virtually every aspect of immune response to an

antigen, Elson, *et al*., (1999).

2.12.1. Cholera toxin and *E. coli*-labile toxin

The two toxins have about 80% sequence homology and exhibit significant immunologic cross-reactivity. The holotoxin consisting of A and B subunits is required for adjuvant effects. Cholera toxin (CT) binds to M cells and to the GM_1-ganglioside receptors on the mucosal epithelium. CT has been shown to enhance the immunogenicity of relatively weak mucosal antigens when conjugated or mixed with the antigen and delivered via the mucosal route. The adjuvant effects are seen on serum IgG and mucosal IgA responses to unrelated antigens administered orally at the same time. Either free or conjugated CTB subunit (CTB) can act as an adjuvant in intranasal immunization, Taylor, (1995).

2.12.2. Vitamin D3

It was first demonstrated in mice that an intramuscular, subcutaneous or intradermal, Enioutina, *et al*., (2000), immunization with a microbial antigen and aluminum hydroxide as the adjuvant induces a mucosal immune response when 1α, $25(OH)_2$, D_3, (vit D3, calcitroil), the active metabolite of vit D3 is added as an additionally immunomodulator. Mice show enhanced antigen specific IgG and IgA, in serum and all tested mucosal secretions (tears, oral, vaginal and colorectal secretions), Daynes, *et al*., (1996).

2.12.3. Avridine

A lipoidal amine, functions by facilitating uptake and release of

antigens in the macrophages in mucosal tissue, with subsequent production of immunoregulating cytokines, Taylor, (1995).

2.12.4. Other adjuvants

Other adjuvants may improve mucosal contact with antigens with or without enhancement of the release of immunoregulatory, cytokine, low-oil emulsion, lipid A and glycerol, Taylor, (1995).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Bacteria

Three field isolates of *P. multocida* (224, 222 and 225) were obtained from the freeze-dried stock of the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum. They were originally isolated from outbreaks of bovine Haemorrhagic Septicaemia (HS) in Halfa and Nyala. They were preserved lyophilized at -20° C. A lyophilized *P. multocida* serotype B: 6 were kindly provided by Central Veterinary Research laboratories. Freeze-dried cultures were reconstituted in nutrient broth and incubated at 37°C for 24 hours; growth was checked and tested for purity and the cultures were kept in slants of blood agar.

3.2. Laboratory animals

3.2.1. Rabbits

Twenty four rabbits were purchased from the local market in Omdurman. Animals were kept at the animal housing of the Department and allowed to adapt for 2 weeks. All rabbits were prophylactically treated with antibiotic and anthelmintic (Albendazole, AVICO). Food and water were supplied and later divided into subgroups each of eight rabbits.

3.2.2. Cattle

Thirty head of cattle in a traditional farm in White Nile state, all

animals were first given prophylactic dose of antibiotic, anthelmintic, and they were divided into three equal groups.

3.3. Sterilization

3.3.1. Flaming

It was used to sterilize wire loops, needles and scalpels.

3.3.2. Red heat

It was used to sterilize wire loops, points and searing spatulas by holding them over bunsen burner flame until became red-hot.

3.3.3. Hot air oven

It was used to sterilize glass wares such as test tubes, graduated pipettes, flasks, forceps and cotton swabs. The holding period was one hour at 160°C.

3.3.4. Moist heat (autoclave)

Autoclaving at 121˚C for 15 minutes was used for the sterilization of media and plastic wares. Autoclaving at 115˚C for 10 minutes was used for sterilization of sugar containing media.

3.4. Bacteriological media

The media utilized in the study constituted the essential nutrients for the growth and maintenance of *P. multocida*. They were principally employed to isolate, modify or to characterize and identify the strains of *P. multocida*. The ingredients incorporated in the media were dissolved by heating with constant stirring by aid of a magnetic stirrer. NaOH and HCL were used to adjust the pH. Media were checked for sterility by overnight incubation at 37^{o}C and for vitality by inoculation

of a positive control and unless otherwise stated they were preserved at 4ºC.

3.4.1. Blood agar

It was the main media used to grow *P. multocida* during the study. Forty grams of blood agar base No.2 (oxoid) were suspended in 1 liter of distilled water, dissolved by boiling, mixed and sterilized. The medium was cooled to 50°C and defibrinated sheep blood was added aseptically to give final concentration of 10%, mixed gently and distributed as 15 ml into sterile Petri dish. The poured plates were allowed to solidify at room temperature on flat surface.

3.4.2. Nutrient agar

To one liter of nutrient broth (oxoid), 15g agar were added, it was dissolved by boiling and sterilized, cooled to bout 50°C and distributed in 15 ml amount per plate. The poured plates were left to solidify at room temperature on a leveled surface.

3.4.3. Nutrient broth

Thirteen grams of nutrient broth powder (oxoid) were dissolved in one liter of distilled water, mixed well, distributed in 3 ml amount into clean test tubes and sterilized.

3.4.4. Brain heart infusion broth (Oxoid)

One liter of medium was prepared by dissolving 37g of powder (conained 12.5 g calf brain infusion solids, 5g beef heart infusion solids, 10g proteose peptone, 5g Nacl, 2g Dextrose and 2.5g disodium hydrogen phosphate) in one liter of distilled water. The pH was

adjusted to 7.4 and the mixture was distributed into Bijou bottles (4ml/ bottle) allowed to cool and stored at 4 °C.

3.5. Solutions

3.5.1. Phosphate buffer saline (PBS)

Constituted of three solutions was mixed to form PBS. These were Solution (A) which was prepared by dissolving 16 g NaCl, 0.4 g KCl, 2.3 g disodium hydrogen phosphate into 1500 ml of deionized distilled water. Solution (B) which was prepared by dissolving 0.42 6g magnesium chloride hydrous into 200 ml deionized distilled water. Solution (C) which was prepared by dissolving 0.264g calcium chloride in 200 ml deionized distilled water.

The three solutions were separately autoclaved at 121°C for 15 minutes and allowed to cool at room temperature. Solution B was aseptically added to solution A followed by solution C and the final volume was brought to two liter by the addition of sterile distilled water.

3.5.2. Normal saline

It was prepared by dissolving 8.5g NaCl in one liter of distilled water and autoclaving at 121° C for 15 minutes.

3.5.3. Saturated ammonium sulfate (SAS)

A saturated solution of ammonium sulfate (SAS) was made by dissolving 760 g of pure ammonium sulfate in one liter of distilled water and sterilization at 121°C for 15 minutes. It was then allowed to stand at room temperature for several days until used.

3.6. Preparation of the vaccine antigens

The antigens used in this study were haemorrhagic septicaemia vaccine (whole formalin-killed P. *multocida* serotypes B and E), a cell lysate of *P. multocida* serotype B and toxoid prepared from *P. multocida* culture.

3.6.1. Haemorrhagic septicaemia vaccine

It was prepared according to the protocol of vaccine production (without adjuvant) in Central Veterinary Research Laboratories (Soba). They were all propagated in the Gottingen bioreactor (fermenter) under the optimized physical growth conditions, temperature 37.5ºC, pH 7.4 and stirring 300 rpm, for vaccine production, as a rule, all production steps of vaccine were done under strict sterile conditions, determinated by Elbashir, (1993).

3.6.2. Preparation of cell lysate

P. multocida serotype B:6 was grown in nutrient broth for 48 hours at 37°C. 100 ml culture were harvested by centrifugation at 3000 rpm for 15 minutes at 4°C and the packed cellular mass was washed three times with phosphate buffer saline pH 7.0. The cells were then resuspended in the same buffer and subjected to ultrasonic disruption (Sonnicator) (MSE-England) 18000 rpm for 10 minutes at 4°C Mortenson *et al.,* (1962). Cell debris and unbroken cells were removed by centrifugation at 3000 rpm for 15 minutes and the supernatant was stored frozen until used.

3.6.3. Culture of the organism and toxin harvest

P. multocida serotype B: 6 was grown on blood agar for 24 hours at 37°C in candle jar (5-10% C_2O). After incubation single colonies were used to seed bottles of brain heart infusion broth which were incubated at 37°C for 3 days. The broth cultures were removed from the incubator and allowed to stand overnight at 4°C the bacterial cells settled on the bottom of the bottles during this period, leaving a relatively clear supernatant broth, the supernatant were carefully decanted into glass centrifuge cups and centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was then filtrated through 0.45 um millipore filter, Suleiman, (1995).

3.6.3.1. Partial purification of the toxin

The exotoxin was precipitated according to the method of Suleiman, (1995), 122 ml of the saturated solution of ammonium sulphate (SAS) was added slowly drop-wise to 100 ml of the culture filtrate thus effecting a 55% ammonium sulphate saturation of the filtrate. The procedures were carried out at room temperature except the flask containing the toxin which was immersed in an ice bath during the precipitation procedure. After the SAS was added the mixture was allowed to stand overnight at 4°C, the precipitate was then removed by centrifugation at 5000 rpm at 4°C. It was then dissolved in PBS containing 0.02% sodium azide as preservative. The final volume was approximately 10 % of original culture, this solution was transferred to cellulose dialysis tubing and dialysed for 36 hrs against DW at 4°C. Part of the dialysed material containing the

partially purified toxin was used to test it's possible dermonecrotic activity while the rest was formalinized (0.3%) and kept at 4°C and stored frozen until used.

3.7. Dermonecrotic activity

The ability of the crude toxin to induce dermonecrosis in a rabbit skin was assayed by inoculation of 100 *u*l of crude toxin intradermally into two rabbits. Acontrol group of two rabbits was inoculated with sterile normal saline. Animals were observed for 48 hours for development of injection site reactions.

3.8. Preparation of *P. multocida* toxoid

0.3% formaldehyde was added to the partially purified toxin and was kept at 4°C.

3.9. Mucosal adjuvants

The safety of *Nigella sativa* seed oil as mucosal adjuvants was initially tried in rabbits. Two rabbits were used and each received 100 and 250 *u*l subcutaneously of crude oil.

Pea-nut oil mixed (1:1 v/v) with Freund's incomplete adjuvant was also experimented as a possible safe mucosal adjuvant in 50 *u*l per animal (rabbits and cattle).

3.10. Immunization of rabbits

3.10.1. Bacterin vaccine

The bacterin was mixed with the mucosal adjuvant and the mixture was placed in a water bath at 37°C for 15 minutes. 100 *µ*l of the preparation was administered intranasally (3 rabbits) and

subcutaneously (3 rabbits) and all rabbits received a booster dose on day 24 post initial inoculation. A control group of two rabbits received normal saline through the same routes as for the treated groups.

3.10.2. Cell lysate

The cell lysate was mixed with the mucosal adjuvant and the mixture was placed in a water bath at 37°C for 15 minutes. 100 μl of the preparation was administered intranasally (3 rabbits) and subcutaneously (3 rabbits) and all rabbits received a booster dose on day 24 post initial inoculation. A control group of two rabbits received normal saline through the same routes as for the treated groups.

3.10.3. Toxoid

The toxoid was mixed with the mucosal adjuvant and the mixture was placed in a water bath at 37°C for 15 minutes. 100 μl of the preparation was administered intranasally (3 rabbits) and subcutaneously (3 rabbits) and all rabbits received a booster dose on day 24 post initial inoculation. A control group of two rabbits received normal saline through the same routes as for the treated groups.

3.11. Samples collection from rabbits

Blood was collected from jugular vein; serum was separated and stored at -20°C. Nasal washings were obtained by tilting the rabbit's head to one side and infusing 1ml of sterile phosphate buffer saline into the nares and collecting drops which were sneezed onto a sterile Petri dish, this process was repeated for the other side the fluid collected and frozen at-20°C Jarvenin, *et al.,* (1998).

Blood and nasal washing samples were collected (i) prior to

inoculation (days 0), (ii) 1 week following initialed inoculation (7, 14 and 21), (iii) 1 week after booster dose.

3.12. Challenge inoculation of rabbits

Cultivation for viable counts was done on blood agar and viable bacteria counts were performed by serial ten fold dilution using the Miles and Misra, (1938). Challenge of vaccinated rabbit groups and unimmunized control groups was carried out on day 35 post vaccination by intranasal administration of dose of $2.5x10^6$ CFU/ml. The dose was chosen based on results of a previous study Jarvinen *et, al.,* (1998).

3.13. Immunization of cattle

3.13.1. Group 1

The bacterin was mixed with the mucosal adjuvant (1:1 v/v) and the mixture was placed in a water bath at 37°C for 15 minutes. 0.5 ml of the preparation was administered subcutaneously (8 cattle), cattle received a booster dose on day 24 post initial inoculation. A control group (2 cattle) received normal saline through the same route as for the treatment group.

3.13.2. Group 2

A dose of 0.5 ml of the antigen described above was inoculated intranasally (8 cattle), the cattle received a booster dose on day 24 after post- vaccination. Control group (2 cattle) received normal saline via the same route of the treatment group.

3.13.3. Group 3

This group served as control group, received 1ml of the bacterin (Final product), a recommended dose according to Central Veterinary Research Laboratories (Soba). The group was vaccinated with the bacterin without the mucosal adjuvant. 1ml of the bacterin was administered subcutaneously (8 cattle), the cattle received a booster dose on day 24 post initial inoculation. A control group (2 cattle) received normal saline through the same route as for the treatment group.

3.14. Sample collection

Blood was collected from jugular vein; serum was removed and stored at -20C. Nasal samples were obtained by swabbing both nares of cattle and the swabs were immersed in tubes containing phosphate buffer saline which was kept at -20°C until processing.

Blood and nasal swabs samples were collected (i) prior to inoculation (days 0), (ii) 1 week following initialed inoculation (7, 14, 21 and 24), (iii) 1 week after booster dose.

3.15. Indirect haemagglutination test (IHA)

This was originally performed using antigen-sensitized human type O red blood cells (RBCs) Carter, (1955), but more recently sheep red blood cells (RBCs) have been used Sawada, (1982). The antigen is prepared as follows;

Bacteria cultured on blood agar and incubated overnight at 37°C. The growth is harvested in 3 ml normal saline containing 0.3% formalin. This suspension is then heated at 56°C for 30 minutes, centrifuged at

3000 rpm for 15 minutes at 4°C, and the clear supernatant was stored at -20°C. This is used as the antigen extract. Sheep blood was collected aseptically into anticoagulant and centrifuged at 500 rpm for 10 minutes. The packed red blood cells (RBCs) are washed three times in sterile normal saline. The antigen extract described above was used to sensitize the red blood cells (RBCs) or absorbed on to the red blood cells (RBCs). This is done by adding 15 volume of the antigen extract to the red blood cells (RBCs) and incubating the mixture for one hour at 37°C with frequent shaking. The sensitized red blood cells (RBCs) are recovered by centrifugation, washed three times in sterile normal saline, and made up to a final 1% suspension in normal saline.

The test was carried out in microtitre plates, and was performed as described by Cho, *et al.,* (1976) as follows:

1) The extract of the strain was prepared as described above and used to sensitize the sheep red blood cells (RBCs).
2) Using two separate rows of wells the first wells are filled with 180 *u*l normal saline followed by 100 *u*l in the next six wells or more.
3) The samples of serum and nasal wash were each separately diluted in each row by adding 20 *u*l of the serum to the first well and mixing with a pipette. From this well 100 *u*l were transferred to the next well, mixed, and the process carried on until well seven. This constitutes 1/10 dilution in the first well and a doubling dilution thereafter.
4) All the wells are each filled with 100 *u*l of antigen-adsorbed/ sensitized red blood cells (RBCs), shaken slightly and left at room

temperature.

By the addition of the sensitized blood, the serum and nasal wash dilution in the wells are a doubled, e.g. 1/20 in well one, 1/40 in the second, and so on.

A positive, negative and saline control are included for each test run.

5) The first reading is taken after two hours and a final reading after 18 hours. A coarse agglutination of the red blood cells (RBCs) along the sides of the concave wells is taken as a positive reading, and the formation of a button at the center of the wells as negative. An arbitrary score of 1-4 is given depending on the size of the agglutination . Indirect haemagglutination test (IHA) can be used for typing unknown strains, the test itself is more efficient when dealing with serotypes B and E and is more reliable as quantitative tests against these strains .

CHAPTER FOUR
RESULTS

4.1. Mucosal adjuvant

250 and 100 *µL* of *Nigella sativa* crude oil injected subcutaneously and intranasally resulted in rough coat, lacrimation, fever and death occured within 24 hour. Due to the toxicity of *Nigella sativa* crude oil in rabbit Zaoui *et al.*, (2002), it was excluded from experimentation as a mucosal adjuvant.

Pea-nut oil mixed with Freund incomplete adjuvant (v/v 1:1) employed as a possible safe mucosal adjuvant in rabbits proved to be safe and hence it was used to deliver the antigens in cattle and rabbits.

4.2. Dermonecrotic activity of the partially purified toxin

Filtrated culture supernatants of *P. multocida* injected intradermally in rabbits caused a local inflammation with no dermonecrosis that returned to normal three days post injection, (Fig 5).

4.3. Immune response of rabbits

4.3.1. Bacterin vaccine

Nasal lavage from immunized rabbits and controls were tested for mucosal antibody by the indirect heamagglutination test. The highest titer (20, 20 and 10), was recorded in samples tested on day 21 post primary immunization. The antibody titer increased after boosting the animals Results are shown in table 1 fig 1.

Results of the indirect haemagglutination test for serum samples

of rabbits immunized with bacterin are shown in table 1. The highest titer (40, 80, and 40), was obtained in samples taken at day 21 post vaccination. The antibody titer doubled after the booster dose.

4.3.2. Cell lysate

The indirect heamagglutination test of nasal washes from vaccinated rabbits and unvaccinated control group detected mucosal antibodies. High titer (10, 20 and 10) was reported in samples taken on day 21 and after boosting. Results are shown in table 2 and fig 2.

Serum samples from cell lysate inoculated rabbits and controls group were tested by the indirect heamagglutination test. The highest titers were recorded in samples tested on day 21 post primary immunization and after booster dosing (Table 2).

4.3.3. Toxoid

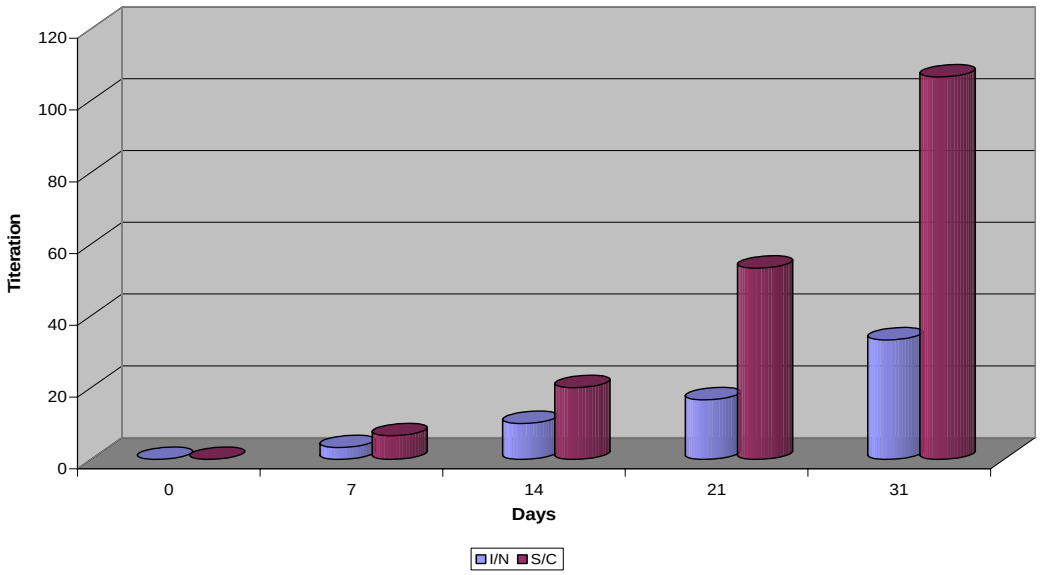
The results of the rabbit group nasal lavages taken from immunized rabbits and control group and test by the IHA documented the presence of mucosal antibodies. The highest titers were recorded in samples taken on day 21 (20, 10, and 10). The antibody titer increased after booster dose. Results are illustrated, (Table 3 and Fig 3).

The highest titers subcutaneously for toxoid vaccinated group of rabbits were reported on sera sampled on day 21 and after booster inoculation (table 3).

Table (1). Results of indirect haemagglutination test of rabbit groups vaccinated with *P. multocida* bacterin.

Route of administration	Rabbits	Day 0	Day 7	Day 14	Day 21	Day 24	Day 31
Intranasal	1	0	0	10	20	Booster	40
	1	0	10	10	20	Booster	20
	1	0	0	10	10	Booster	40
	Control	0	0	0	0	N. Saline	0
Subcutaneous	1	0	10	20	40	Booster	80
	1	0	10	20	80	Booster	160
	1	0	0	20	40	Booster	80
	Control	0	0	0	0	N. Saline	0

FIG (1) Average of IHA of group of rabbits immunization with bacterin



4.4. Challenge exposure of vaccinated rabbits.

4.4.1. Bacterin

The survival rate of intranasally and subcutaneously bacterin immunized groups of rabbits was 100% while the entire control group succumbed within 24 hour (table 4).

4.4.2. Cell lysate

67% was the rate of survival for the rabbit group vaccinated intranasally with cell lysate. All animals in the control group died after 24 hr post challenged. All rabbits in the subcutaneous immunized group survived the challenged inoculation while the control group died within 24 hours, (table 4).

4.4.3. Toxoid

Survival rate of rabbits group immunized intranasally with toxoid had a 67%. All animals in the control group succumbed within 24 hour after challenged. Group of rabbits vaccinated subcutaneously had a 100% survival rate (table 4).

4.5. Immunization of cattle

4.5.1. Group 1

Serum sample from immunized cattle and controls were tested for antibody by indirect haemagglutination test. The highest titer was recorded in samples tested on day 24 post primary immunization. The antibody titer increased after boosting the animals. Results are shown in table 5 and fig 4.

Table (2). Results of indirect haemagglutination test of rabbit groups immunized with *P. multocida* cell lysate.

Route of administration	Rabbits	Day 0	Day 7	Day 14	Day 21	Day 24	Day 31
Intranasal	1	0	0	10	10	Booster	20
	1	0	0	10	20	Booster	40
	1	0	0	10	10	Booster	40
	Control	0	0	0	0	N. Saline	0
Subcutaneous	1	0	10	10	20	Booster	40
	1	0	0	10	20	Booster	80
	1	0	10	20	40	Booster	160
	Control	0	0	0	0	N. Saline	0

FIG (2). Average of IHA of group of rabbits inoculation with cell lysate

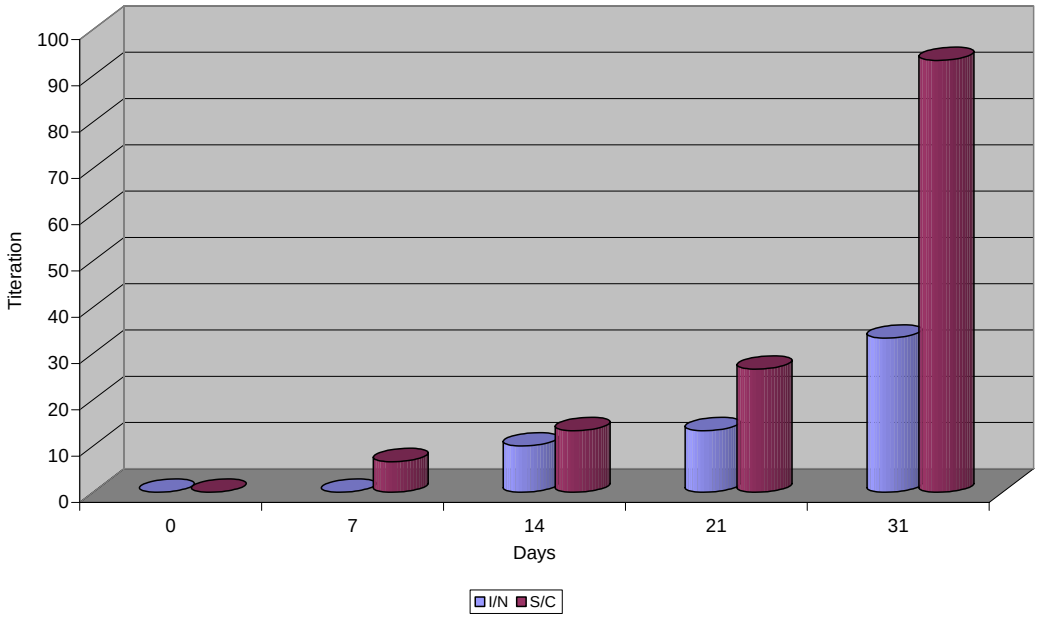
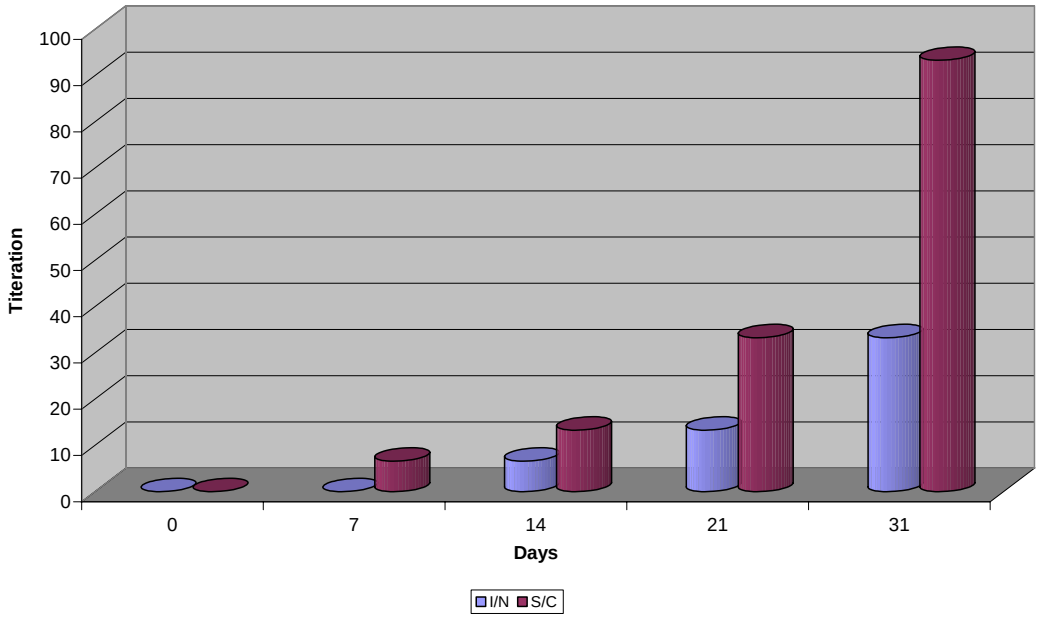


Table (3). Results of indirect haemagglutination test of rabbit groups inoculated with *P. multocida* toxoid.

Route of administration	Rabbits	Day 0	Day 7	Day 14	Day 21	Day 24	Day 31
Intranasal	1	0	0	10	20	Booster	40
	1	0	0	0	10	Booster	20
	1	0	0	10	10	Booster	40
	Control	0	0	0	0	N. Saline	0
Subcutaneous	1	0	10	10	20	Booster	40
	1	0	0	20	40	Booster	160
	1	0	10	10	40	Booster	80
	Control	0	0	0	0	N. Saline	0

Fifg (3). Average of IHA of group of rabbits vaccinated with toxoid



4.5.2. Group 2

The indirect heamagglutination test of nasal swab from vaccinated cattle and unvaccinated control group detected for mucosal antibodies. High titer was reported in samples taken on day 24 and after boosting animals (table 5).

4.5.3. Group 3

Results of the indirect haemagglutination test for serum samples of cattle group immunized with bacterin are shown in table 5. The highest titer was obtained in samples taken at day 24 post vaccination. The antibody titer rised after the booster dose, see table 5 and fig 4.

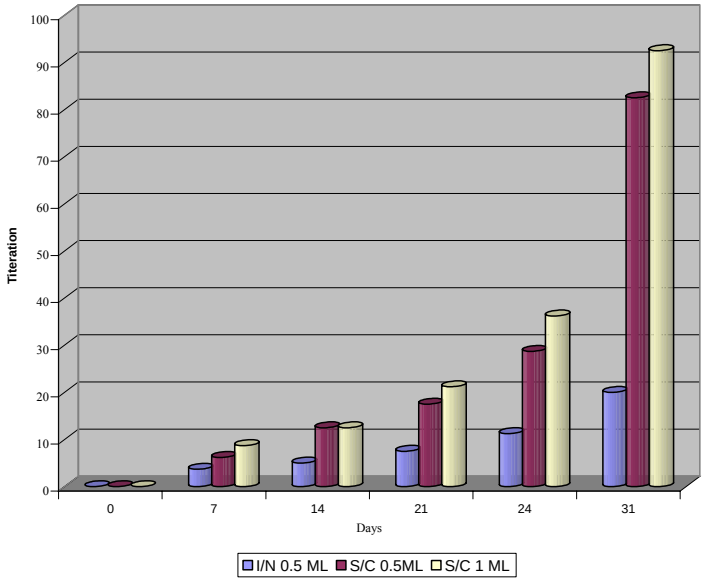
Table (4). Results of challenge inoculation in rabbits immunization groups.

Vaccine	Route of administration	No. of rabbits	No. of dead	No. of live
Bacterin	Intranasal	3	0	3
	Subcutaneous	3	0	3
Cell lysate	Intranasal	3	1	2
	Subcutaneous	3	0	3
Toxoid	Intranasal	3	1	2
	Subcutaneous	3	0	3

Table (5). Average results of indirect haemagglutination test of eight cattle vaccinated with *P. multocida* bacterin

Route of administration	Day 0	Day 7	Day 14	Day 21	Day 24	Day 31
I/N (0.5 ml)	0	3.75	5	7.5	11.25	20
S/C (0.5 ml)	0	6.25	12.5	17.5	28.75	82.5
S/C (1 ml)	0	8.75	12.5	21.25	36.25	92.5

fig (4) Average of indirect haemagglutination test of three cattle groups (8 cattle each) vaccinated with *P. multocida* bacterin



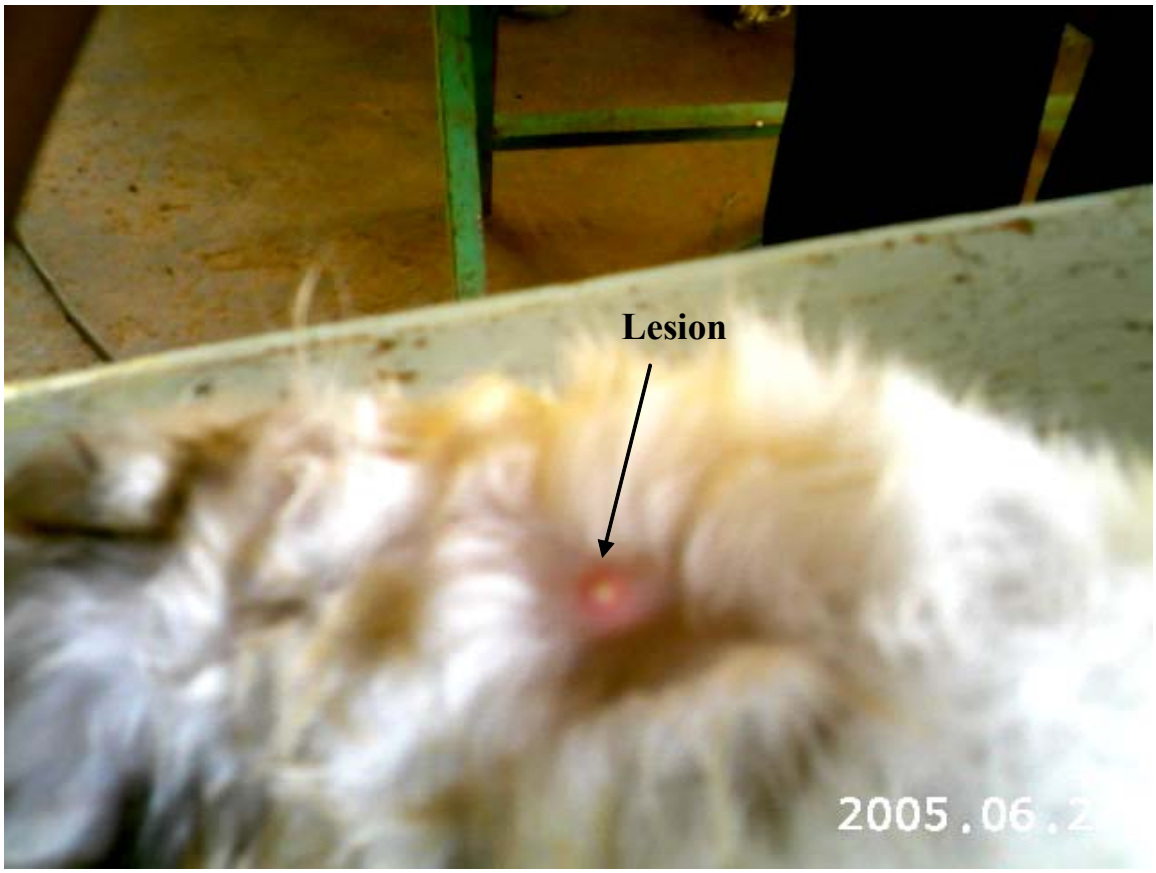


Fig (5): Dermonecrotic activity of the partially purified *P. multocida* toxin

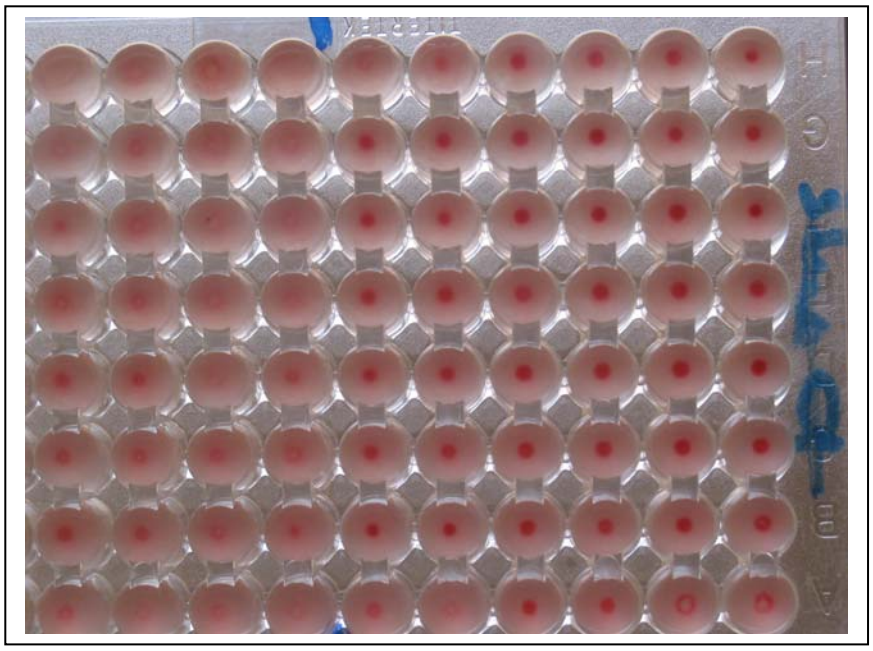


Fig (8): Indirect haemagglutination test in microtitre plate.

CHAPTER FIVE
DISCUSSION

The present study was conducted to compare between efficacy of intranasal vaccination and subcutaneous vaccination in protecting rabbits and cattle against pneumonic pasteurellosis with different vaccine preparations.

Mucosal adjuvants are importance in the delivery of vaccines to mucosal surface, these adjuvant should be non-irritant and have the minimal local side effect. In the study the *Nigella sativa* oil used as mucosal adjuvant was found toxic and lethal to rabbits when applied intranasally or subcutaneously. The exact mechanism of toxicity in rabbits remains to be clarified and no previous studies were reported in the literature describing the toxicity of *N. sativa* oil in rabbits.

Furthermore, a mixture Freund's incomplete adjuvant and pea-nut oil (1/1 v/v) was experimented was also were also as mucosal adjuvants in rabbits and no adverse reactions were reported, hence it was used in the present study to deliver the different vaccine preparations that included a bacterin, a toxoid and a cell lysate each of which was used to vaccinate groups of rabbits and cattle via the intranasal and subcutaneous route.

Filtrated culture supernatants of *P. multocida* injected intradermally in rabbits caused a local inflammation with no dermonecrosis, this finding in similar to the results reported by Dhanda, (1959).

Immunity in vaccinated animals was measured indirectly by

determination of the antibody titer using the indirect haemagglutination test and directly by challenge of vaccinated animal using viable *P. multocida*.

The bacterin vaccine was experimented in two groups of rabbits and it was administered intranasally and subcutaneously, using pea-nut-freund's incomplete adjuvant in both route. The indirect haemagglutination titers were greater in the subcutaneously inoculated group, compared with the titer of intranasally inoculated group. The lower titre recorded in the intranasally vaccinated groups might be the fact that the nasal lavage covered only small part of the respiratory mucosal surface hence the titre measured was not quantitative for the elicited mucosal immune response. The two vaccinated groups showed 100% survival rate when challenged with $2.5x10^6$ CFU/ml of *P. multocida*. And this result further substantiated that the intranasal vaccination affected protection in rabbits comparable to that elicited following subcutaneous vaccination. The results of bacterin vaccination in rabbits of the present study was in agreement with that reported by (Elbashir, 1993) who determined the immunogenic quality of the HS bacterin vaccine in group of rabbits administered through the subcutaneous route.

The antibody titer of the cell lysate vaccinated rabbit groups was higher in the subcutaneous route compared to the intranasal application, and might be explained as above of the bacterin. The survival rate of the two groups following challenge with $2.5x10^6$ CFU/ml was 100% for subcutaneous vaccinated group and 67% for intranasal vaccinated group. Results of this study clearly demonstrated

that cell lysate vaccine of *P. multocida* administered subcutaneous into rabbits were more efficient in eliciting antibody immune response and more protective against challenge exposure when compared to the same vaccine inoculated via the intranasal route.

In the toxoid vaccinated rabbit groups, the titers were greater in the subcutaneously inoculated group compared to the intranasally inoculated group. The survival rate of the groups following challenge with $2.5x10^6$ CFU/ml was 100% and 67% for subcutaneously and intranasally vaccinated groups, respectively, indicating that the toxoid administered via the subcutaneous route affected better protection than that given intranasally. These results are partially consistent with the findings of Jarvinen *et al*., (1998) who vaccinated rabbits with a toxoid and potassium thiocyanate extract from cultures of *P. multocida* and demonstrated a 100 % protection in the two groups of rabbits immunized intranasally and subcutaneously.

The bacterin vaccine was experimented in two groups of cattle (each 10 cattle). via subcutaneous and intranasal routes with pea-nut plus Freund's incomplete adjuvant. Results of indirect haemagglutination titers were greater in the subcutaneously inoculated group compared to intranasally immunized group.

The third group of cattle (10 cattle) which was immunized with 1ml bacterin subcutaneously according to the program of vaccination of cattle against haemorrhagic septicaemia in the field. Showed indirect haemagglutination titers greater than the titres of group of cattle immunized via the intranasal route. This finding is in consistency with that of (Elbashir, 1993) who used a bacterin vaccine

produced in a continuous cultivation system. And such vaccines were shown to be antigenically superior to produced in static cultures.

In this study mucosal immunity to *P. multocida* resulted by vaccination with a bacterin, cell lysate and toxoid were in general less protective than subcutaneous route and this might be due to the fact that we used only one adjuvant necessitate in the future experimentation of other mucosal adjuvant which might confer better protection than the pea-nut-ferund's incomplete adjuvant used in the present study.

Even though intranasal immunization with bacterin is an effective way to control infection, the method of vaccine delivery might be difficult to practice, especially when vaccinating a large number of animals. However, the efficacy of mucosal vaccination suggests that, it may eventually be possible to deliver these antigens by alternative routes, such orally, to induce mucosal immunity in respiratory tracts.

The titration of nasal swabs and nasal wash samples in cattle and rabbits respectively represents only small level titers because; the titers of the nasal samples may not represent the total respiratory tract surfaces, but despite this measurable antibody titre was detected.
Nasal swabs are not a suitable technique for measuring secretary IgA, because the collection of nasal samples limited in nasal mucosa area of respiratory tracts.

In conclusion the study documented that *P. multocida* preparations administered to the local nasal mucosa elicited protective mucosal immune response. In addition results of the present

investigation showed that a mixture of ferund's incomplete adjuvant and pea-nut oil demonstrated could act as a safe mucosal adjuvant to deliver antigens onto mucosal surfaces.

RECOMMENDATION

According to results of the present study the following is recommended:

- More studies on *P. multocida* should be conducted to experiment the delivery of vaccines via the intranasal rout rather than other routes.
- Safe mucosal adjuvant should be explored to deliver P. *multocida* vaccines.
- Design of new vaccine formulations that will be elicit protective immunity at mucosal surfaces.
- More research should be done to test the efficacy of the current bacterin vaccine used to protect the national herd.

REFERENCES

Annosa, V. O., Isoun, T. T., 1975. An outbreak of haemorrhagic septicaemia in Holstein cattle in Nigeria : possible role of associated factors.Bull. Anim. Health pord. Afric. **23**: 337-340..

Annual report :, Sudan veterinary services (1933, 39, 43 and 47).

Anonymous, 1933, 1959. Annual reports of the Sudan veterinary services.

Anonymous, 1979. Proc. FAO/APHCA, Workshop on haemorrhagic septicaemia, Colombo, Sri Lanka.

Baba, T., (1984). Cell mediated immune protection in chickens against *P.multocida.*Res.Vet.Sci.**36:**225-230

Baillie, W.E., Stowe, E.C., Schmitt, A.M., (1978). Aerobic bacterial flora of oral and nasal fluids of canines with reference to bacteria associated with bites. J. clin. Microbiol. 7: 223.

Bain, R. V. S. (1963). Addendum to the genus *Pasteurella*. In Namioka and Murata (1961). Serological studies on *P. multocida* iv) Type distribution of the organisms on the bases of their capsule and O-groups. Cornell Vet., **53:** 41-53.

Bain, R. V. S. (1963). Haemorrhagic septicaemia. Agricultural studies, No. 62, p. 78, Rome, FAO.

Bain, R.V.S., (1954). Studies on haemorrhagic septicaemia of cattle. I. Naturally acquired immunity in Siamese buffaloes. Brit. Vet. J. **110:** 481-484.

Bain, R.V.S., (1957). The problem of the haemorrahagic septicaemia in cattle. Ceylon. Vet. J. **5:** 2-7.

Banerji, T.P. Mukherjee, (1953). Nutritional requirement of *Pasteurella septica*. Current. Sci. **22**: 177-178.

Bastianello, S.S., Jonker, M.R., (1981). A report on the occurrence of septicaemia caused by *P. multocida* type 1 in cattle from southern Africa. J. South Africa. Vet. Ass. **52:** 99-104.

Bernstein, J. M., J. Gorfien, and P. Brandtzaeg **(**1999). The immunobiology of the tonsils and adenoids, p. 1339-1362. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee (ed.), Mucosal immunology, 2nd ed. Academic Press, New York, N.Y.

Bierer, B.W., (1969). Comparision of a live drinking water vaccine for fowl cholera in turkeys to a killed drinking water vaccine and to five injected commercial bacterins. Poult. Sci. **48:** 1396-1400.

Bollinger, (1878). Pasteurellosis. Cited by Hudson, J.R. In: Stableforth, A.W., Galloway, I.A. Infectious diseases of animals: Diseases due to bacteria, Vol. 2. Butterworth, London, 421.

Bording, A., Riising, H.J., (1986). Vaccination against atrophic rhinitis. A field study on Tinord. In Proc. 9th Cong. Int. Pig vet. Soc. 245.

Carter, G. R. (1955). A haemagglutination test for the identification of serological types. Am. J. Vet. Res., **16:** 481-484.

Carter, G. R. (1957). Studies on *P. multocida* on identification of Antigenic and colonial characteristics . Am J. Vet. Res., **18:** 210-213.

Carter, G. R. (1959). Studies on *P. multocida* iv) Serological types from species other than cattle and swine. Am. J. vet. Res., **20:** 173-175.

Carter, G. R. (1967). Pasteurellosis. *P. multocida* and *P. hepatica*. Adv. Vet. Sci., **11:** 321-379

Carter, G.R., (1982). Whatever happened to haemorrhagic septicaemia ? J. Amer. Vet. Med. Ass., **180:** 1176-1177.

Carter, G.R., (1984). *Pasteurella, Yersinia,* and *Franciella*. In: Diagnostic Procedures in Veterinary Bacteriology and Mycology. 4th ed. Charles C. Thomas, Springfield, 111-121.

Carter, G. R. (1984). Genus *Pasteurella.* In Bergey Manual of Systemic Bacteriology, Vol. 1, Williams and Wilkins, Baltimore, 552-557.

Carter, G. R., De Alwis, M.C., (1989). Haemorrhagic septicaemia. In : Adlam, C., Rutter. J.M., *Pasteurella* and Pasteurellosis. Academic Press, London. 131-160.

Carter, G.R. Theodorides, V.J., Freman, J.F., (1972). Blood concentration study of sulfamethazine in calves infected with *P. multocida* Vet. Med. Small anim. Clin. **67:** 739-642.

Carter, G.R., Bain, R.V.S., (1960). Pasteurellosis (*P. multocida*): a review stressing recent developments. Vet. Rev. Annot. **6:** 105-128.

Carter, G.R., De Alwis, M.C., (1989). Haemorrhagic septicaemia. In Adlam, C., Rutter. J.M., *Pasteurella* and Pasteurellosis. Academic Press, London. 131-160.

Cho H J, Ruhnke H L, Langford E V. (1976). The indirect hemagglutination test for the detection of antibodies in cattle naturally infected with Mycoplasmas. Can J Comp Med.**40:**20–29.

Confer, A. W. ; R. J. Panciera; R. w. Fulton; M. J. Gentry and J. A. Rumage (1985). Effect of vaccination with live or killed *P. haemolytica* on resistance to experimental bovine pneumonic pasteurellosis . Am. J. Vet. Res., **46:** 342-347.

Conley, M. E., and D. L. Delacroix**., (**1987). Intravascular and mucosal immunoglobulin A: two separate but related systems of immune defense. Ann. Intern **106:** 892-899.

Dasso, J. F., and M. D. Howell **(**1997). Neonatal appendectomy Impairs mucosal immunity in rabbits. Cell. Immunol. **182:** 29-37.

Dawkins, H.J.S., Johnson, R.B., Spencer, T. L., Patten, B.E., (1990). Rapid identification of *P. multocida* organisms responsible for haemorrhagic septicaemia using an enzyme-linked immunosorbent assay (ELISA). Res. vet. Sci., **49:** 261-267.

Dawkins, H.J.S., Ramadani, Johnson, R.B., Spencer, T. I., (1991). Haemorrhagic septicaemia correlation of vaccinal antibody responses in mice with protection against *P. multocida* strain M1404. Vet. Microbiol. 27, 309-326.

Daynes, R.A., Enioutina, E.Y., Butters, M. H.H., Mc Gee, Z.A., Araneo, B.A., (1996). Induction of common mucosal immunity by hormonally immunodulated peripheral immunization. Infect. Immun. **60:** 1100-1109.

De Alwis, M.C.L., Vipulasiri, A. A., (1980). An epizootiological study of haemorrhagic septicaemia in buffaloes and cattle in Sri Lanka. Ceylon vet. J. **28:** 24-35.

De Alwis, M.C.L., (1981). Mortality among cattle and buffaloes in Sri Lanka due to haemorrhagic septicaemia. Trop. Anim. Hlth. Prod. **13:** 195-202.

De Alwis, M.C.L, (1984). Haemorrhagic septicaemia in cattle and buffaloes. Rev. sci. tech.Off. int. Epiz. **3:** 707-730.

De Alwis, M.C.L., Wijewardanda, T.G. Vipulassiri, A.A., (1986a). livestock production diseases in the tropics. Proc. 5th Conf. inst. Trop. Vet. Med., Kuala Lumpur, Malaysia, vol. **1:** 99-100.

De Alwis, M. C. L. (1987) Serological classification of *P*. *multocida*. Vet. Rec., 121, 44.

De Alwis, M.C.L., (1987). Short communication serological classification of *P. multocida*. Vet. Rec. 121, 44.

De Alwis, M.C.L., (1990). Persistence of the carrier status in Haemorrhagic septicaemia (*P. multocida* serotype B: 6 infection) in buffaloes. Trop. Anim. Hlth. Prod. **22:** 185-194.

Dhanda, M.R., (1959). Immunological studies on *P. septica* III. Indirect tests as indications of immunity in haemorrhagic septicaemia. Ind. J. vet. Sci. **29:** 30-46.

DiGiacomo, R. F., B. J. Deeb, W. E. Giddens, Jr., B. L. Bernard, and M. M. Chengappa (1989). Atrophic rhinitis in New Zealand White rabbits infected with *P. multocida*. Am. J. Vet. Res. **50:** 1460-1465.

DiGiacomo, R. F., C. D. R. Jones, and C. M. Wathes (1987). Transmission of *P. multocida* in rabbits. Lab. Anim. Sci. **37:** 621-623.

Elbashir, S. M., (1993). Production of vaccine against haemorrhagic septicaemia (HS) with Göttingen Bioreactor Technology using local Sudanese *Pasteurella multocida* strains B and E.

Elson, C. O., and M. T. Dertzbaugh **(**1999). Mucosal adjuvants, p. 817-838. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee (ed.), Mucosal immunology, 2nd ed. Academic Press, New York, N.Y.

Enioutina, E.Y., Visic, D., Daynes, R.A., (2000). The induction the systemic and mucosal immune resposes to antigen-adjuvant compositions administered into the skin : alterations in the migratory properties of dendritic cells appears to be important for stimulating mucosal immunity. Vaccine **18:** 2753-2767.

Flatt, R. E. (1974). Bacterial disease : pasteurellosis, p. 194-205. In H. Weisbroth, R. E. Flatt, and A. L. Kraus (ed.), The biology of the laboratory rabbit. Academic Press, Inc., New York, N.Y.

Foged, N. T. (1992). *P. multocida* toxin : the characterization of the toxin and its significance in the diagnosis and prevention of progressive atrophic rhinitis in pigs. APMIS Suppl. **25:** 1-56.

Francis, B. K. T.; H. F. Schels and G. R. Carter (1980). Type E *P. multocida* associated with haemorrhagic septicaemia in Zambia. Vet., Rec ., 107, 6, 135.

Frederiksen, W. (1973). *Pasteurella* taxonomy and nomenclature. Contrib. Micrbiol. Immunol., **2:** 170-176.

Gaertner, D. J. (1991). Comparison of penicillin and gentamicin for treatment of pasteurellosis in rabbits. Lab. Anim. Sci**. 41:** 78-80.

Geering, W. A. (1984). In emergency diseases of livestock. Vol.,1, FAO, Rome.

Gochenour, W.S., (1924). Haemorrhagic septicaemia studies the development of a potent immunizing agent (Natural aggressin) by the use of highly virulent Strains of haemorrhagic septicaemia organisms. J. Amer. Vet. Med. Ass. **18:** 433-441.

Gupta, B.K., (1962). studies on carrier problem in haemorrhagic septicaemia. M.V.Sc. thesis, Punjab University, Chandigarh.

Hassan, A. K. M. and A. A. Mustafa (1985). Isolation of type B *P. multocida* from an outbreak of H.S in camels in the Sudan. Rev. Elev. Med. Vet. Pays. Trop. 38, **1:** 31-33.

Hirsh, N. Maclachan, J. Richard L. and Walker, (2004). Veterinary Microbiology, second edition. Blackwell Iwoa. USA,

Huang Y.T., Chu R.M., Liu R.S., Weng C.N. (1990). Morphologic studies of intrapulmonary airway mucosa-associated lymphoid tissues in swine, Vet. Immunol. Immunopathol. **25:** 13–22 .

Hubbert, W.T., Rosen MN., (1970). *P. multocida* infection due to animal bite. Amer. J. publ. Hlth. **60:** 1103-1108.

Hueppe, F., (1886). Ueber die Wildseuche und ihre bedeutung für die National -ökonomie und die Hygiene. Berl. Klin. Wschr. **44:** 753-758.

Jarvinen, L. Z. Hogenesch, M. A. Suckow, and T. L. Bowersock. (1998). Induction of protective immunity in rabbits by coadministration of inactivated *Pasteurella multocida* toxin and potassium thiocyanate extract. Veterinary Pathobiology, Purdue University, West Lafayette, Indiana 47907.

Jaslow, B. W., D. H. Ringler, H. G. Rush, and J. C. Glorioso. (1981). *Pasteurella* associated rhinitis of rabbits : efficacy of penicillin therapy. Lab. Anim. Sci. **31:** 382-365.

Javed, N. H., I. E. Gienapp, K. L. Cox, and C. C. Whitacre **(**1995). Exquisite peptide specificity of oral tolerance in experimental autoimmune encephalomyelitits. J. Immunol. **155:** 1599-1605

Johnes, T.O., Hussaini, S.N., (1982). Outbreak of *Pasteurella multocida* septicaemia in follow deer. Vet. Rec.110, 451-452.

Johnes, T.O., Hussaini, S.N., (1982). Outbreak of *Pasteurella multocida* septicaemia in follow deer. Vet. Rec. **110:** 451-452.

Joseph, P. G. and Hedger (1984). Serological respones of cattle to Simultaneous vaccination against FMD and HS. Vet. Rec., **114:** 494-496.

Kadymov, R. A.; E. M. Agaeva and Sh. V. Veliev (1987). Serotypes of *P. multocida* isolated from sheep in Azerbaijian. Doklady Vsesoyuzoni Selskok hozyaistvennykh, **1:** 33-35.

Kitt, T., (1885). Über eine experimentelle, der Rinderseuche ähnliche Infections krankheit. Sitzungsber. Ges. Morphophysiol. München, 140-168.

Kobish, M. Pennigs, A., (1986). An evaluation of Nob Vac AR and an experimental atrophic rhinitis vaccine containing *P. multocida* DNt-Toxoid and *Bordetella bronchiseptica* in pigs. Proc. 9th Cong. Int. pig. Vet. Soc. 246.

Krishna, D. And R. K. Kaushik (1965). Studies on an outbreak of acute swine pasteurellosis due to *P. multocida* type B (carter, 1955). Vet. Rec., 77, **15:** 411-416.

Lane, E. P.; N. D. Kock; F. W. G. Hill and K. Mohan (1992). An outbreak of H.S. (septicaemic pasteurellosis) in cattle in Zimbabwe. Trop. Anim. Hlth. Prod., 97-102.

Liu, L. M., and G. G. MacPherson (1993). Antigen acquisition by Dendritic cells: intestinal dendritic cells acquire antigen administered orally and can prime naive T cells in vivo. J. Exp. Med. **177:** 1299-1307.

Lu, Y, S.; S. P. Pakes; L. Massey and C. Stefanu (1987). A potassium thiocyanate extract vaccine prepared from *P. multocida* : A protect rabbits against homologous challenge. Inf. Imm., 55, **12:** 2967-2976.

Lutenberg, B.; R. VanBoxtel; D. Evenberg; M. DeJong; P. Storm & J. Frik (1986). Biochemical and immunological characterization of cell surface proteins of *P. multocida* strains causing atrophic rhinitis in pigs. Inf. Imm., 52, **1:** 175-182.

Martinez, A.; E. Aznar and C. Vina (1987). *P. multocida* in the respiratory tract of calves. Revista de Salud An., **9 :** 7-12.

McGhee, J. R., M. E. Lamm, and W. Strober **(**1999). Mucosal immune responses: an overview, p. 485-506. In P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R.McGhee (ed.), Mucosal immunology, 2nd ed. Academic Press, New York, N.Y.

Mestecky, J. (1987). The common mucosal immune system and current strategies for induction of immune responses in external secretions. J. Clin. Immunol. **7:** 265-276.

Miles, A. A. and Misra, S. S. (1938). The estimation of the bactericidal power of blood. J. Hyg., Camb. 38 : 732.

Mortenson, L,E. Valentine, R.C. and Carnhan, J.E. (1962). An electron transport factor for *Clostridium pasteurianum.* Biochem. Biophys. Res. Commun. **7:** 448-452

Mosier, D. A.; A. W. Confer & R. J. Panciera. (1989). The evolution of vaccines for bovine pneumonic pasteurellosis. Res. Vet. Sci., 47, **1:** 1-10.

Nagy, L. K. & C. W. Penn. (1976). Protection of cattle against experimental Haemorrhagic septicaemia by the capsular antigens of *P. multocida* types B & E. Res. Vet. Sci., **20:** 249-253.

Namioka, S. & D. W. Bruner (1963). Serological studies on *P. multocida* iv) Type distribution of the organisms on the basis of their capsule and O-groups. Cornell Vet., **53:** 41-43.

Namioka, S. & M. Murata (1961). Serological srudies on *P. multocida* i) A simplified method for capsule typing of the organisms. Cornell Vet., **51:** 507-522.

Namioka, S. & M. Murata (1961). Serological studies on *P. multocida* ii) Characteristics of the somatic (O) antigen of the organism. Cornell Vet., **51:** 498.

O.I.E. (1990). Recommended diagnosis technique and requirement for biological products. Rev. sc. Tec. **3:** 589-678.

Pasteur, L., (1880). De lattenuation de virus de cholera des poules. Comp. rend. Acad. Sci. **91:** 673-680.

Quinn, P.J., Markey, M.E., Carter, M.E., Donnelly, W.J. and Leonard, F.C., (2002). Veterinary microbiology and microbial disease. Black well Tokyo 104, Japan.

Rimer, R. B. & K. A. Rhoades (1986). Fowl cholera : Influence of *Bordetella Avium* on vaccinal immunity of turkeys to *P. multocida*. Av. Dis., 30, **4:** 838-839.

Roberts, R. S. (1947). An immunological study of *P. septica*. J. Comp. Pathol. **57:** 261-278.

Ron Y., De Baestelier P., Gordon J., Feldman M. and Segal S. (1981) Defective induction of antigen-reactive proliferating T cells in B cell-deprived mice Eur. J. Immunol. 11: 964.

Sawada, T., Rimler R. B. & Rhoades K. P. (1982). Haemorrhagic septicaemia naturally aquired antibodies against *P. multocida* types B and E in calves in the United States. Am. J. Vet. Res., **46:** 1247-1250.

Shigidi, M. T. A. & A. A. Mustafa (1979). Biochemical and serological studies on *P. multocida* isolated from cattle in Sudan. Cornell Vet., 69, **1:** 77-84.

Shigidi, M. T. A. & A. A. Mustafa (1980). Serological relationships between strains of *P. multocida*. Cornell Vet., 70, **2:** 27-36.

Smith, J.E., (1955). Studies on *P. septica*. 1. the occurrence in the nose and tonsils of dogs. J. comp. Path. **65:** 239-245.

Staats, H. F., R. J. Jackson, M. Marinaro, I. Takahashi, H. Kiyono, and J. R. McGhee (1994). Mucosal immunity to infection with implications for vaccine development. Curr. Opinion Immunol. **6:** 572-583.

Stanley A.C., Huntley J.F., Jeffrey M., Buxton D. (2001). Characterization of ovine nasal associated lymphoid tissue and identification of M cells in the overlying follicle-associated epithelium, J. Comp. Pathol. **125:** 262–270

Steinbrink, K., M. Wölfl, H. Jonuleit, J. Knop, and A. H. Enk **(**1997). Induction of tolerance by IL-10-treated dendritic cells. J. Immunol. **159:** 4772-4780.

Stepkowskii, S. & J. Zarzycki (1986). Studies on the seroaontigenic Properties *P. multocida* including the heat susceptibility of the antigenic componens of the microorganisms. Ann. University.Mar. Cur. Sklod., DD (Medicina veterenaria), **38:** 59-66.

Suleiman, K.M., (1995). Studies on *Corynebacteriu pseudotuberculosis.* M.V.Sc. thesis, University of Khartoum.

Syuto, B. & M. Mstsamoto (1982). Purification of a protective antigen from a saline extract of *P. multocida*. Inf. Imm., 37, **3:** 1218-1226.

Taylor, C. E. 1995. Cytokines as adjuvants for vaccines: antigen-specific sresponses differ from polyclonal responses. Infect. Immun. **63:**3241-3244

Thomas, L. M. (2006). *Pasteurella multocida* Infection Department of Internal Medicine, Division of Infectious Diseases, Medical University of South Carolina

Thomson, R.G., Chandler, S., Savan, M., Fox, M.L., (1975). Investigation of factors of probable significance in the pathogenesis of pneumonic pasteurellosis in cattle. Can. J. comp. Med. **39:** 194-207.

Tschernig, T., W. J. Kleemann, and R. Pabst **(**1995). Bronchus associated lymphoid tissue (BALT) in the lungs of children who had died from sudden infant death syndrome and other causes. Thorax **50:** 658-660.

Watts, J. L., R. J. Yancey, Jr., S. A. Salmon, and C. A. Case. (1994). A 4- year survey of antimicrobial susceptibility trends for isolates from cattle with bovine respiratory disease in North America. J. Clin. Microbial. **32:** 725-731.

Yadav, M. S. & M. L. Ahooja (1986). Thermostability and keeping quality trials in rabbits with oil adjuvant and multi-emulsion oil adjuvant vaccine against haemorrhagic septicaemia. Ind. J. An. Sci., 56, **8:** 833-835.

sZamri-Saad M., Effendy A.W., Israf D.A., Azmi M.L., (1999). Cellular and humoral responses in the respiratory tract of goats following intranasal stimulation using formalin-killed *Pasteurella haemolytica* A2, Vet. Microbiol. **65:** 233–240.

Zaoui A, Cherrah Y, Mahassini n, Alaoui K, Amaraouch H, Hassar M. (2002). Acute and chronic toxicity of *Nigella sativa* fixed oil. Phytomedicine **9:** 69-74.